Spring 5-2014

Population Dynamics of Triticum Mosaic Virus in Various Host Species

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POPULATION DYNAMICS OF TRITICUM MOSAIC VIRUS IN VARIOUS HOST SPECIES

by

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A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Agronomy

Under the Supervision of Professor T. Jack Morris

Lincoln, Nebraska

May, 2014

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University of Nebraska, 2014

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It has been established that RNA viruses should be genetically diverse, due to the high error rate of their RNA-dependent RNA polymerases and the lack of proof-reading capabilities. Plant RNA viruses are not as genetically diverse as expected. Evolutionary factors, such as purifying selection and bottlenecks that favor genetic stability, might be affecting plant viral populations. Otherwise RNA virus populations, with their potential for extreme diversity, might acquire a lethal number of mutations leading to the collapse of the population.

Triticum mosaic virus (TriMV) populations maintained in a controlled greenhouse environment displayed genetic stability. The mutation frequency per nucleotide of TriMV protein 1 (P1) and coat protein (CP) cistrons was discovered to be half that of similar plant viruses. Variations within serial passaged TriMV populations were dominated by singletons within both cistrons examined. Thus, stochastic processes such as bottlenecking are impacting the populations observed. The founding inoculum type sequence was preserved throughout the serial passages therefore some level of genetic stability was being maintained.

The mutation frequencies observed within the CP cistron of two TriMV isolates within wheat, rye, barley, and triticale, were not significantly different,

suggesting that the level of TriMV population diversity is maintained across these hosts. The lack of preference for any particular host examined suggests that both TriMV isolates are equally adapted. The variation of the P1 and CP regions of TriMV populations, horizontally transmitted by wheat curl mites, were determined. The alternative host, jointed goatgrass, caused no shift from the founding haplotype of the P1 or CP within the TriMV populations observed, indicating that the bottlenecks occurring within this host are similar to those within wheat. TriMV populations maintain genetic stability, even with the constraints of genetic bottlenecking by horizontal transfer by wheat curl mites.

The TriMV populations observed maintained genetic stability by the evolutionary forces of selection and genetic bottlenecking. This leads us to conclude that selection and drift are not exclusive, and may occur concurrently within a virus population. This research shows that the combined effect of both forces acting simultaneously on different regions of the genome ultimately regulates the degree of sequence variation within virus populations.

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who have supported and helped me during my time at UNL. I would like to thank my committee members: Dr. Bob Graybosch, Dr. Jack Morris, Dr. Satyanarayana Tatineni and Dr. Gary Hein for all of their assistance and expertise throughout this process. A special thanks goes to the now retired, Dr. Roy French. I would like to acknowledge all those who have given me great input and advice along the way. Special thanks to those who helped me edit and revise my dissertation over and over. To the plant pathology graduate students present and past that have helped me succeed while here at UNL. To the plant pathology staff and faculty, I would like to thank you for your support along the way. Thanks to the USDA-ARS Lincoln unit for their support and friendship over the last several years. To my family and friends who kept encouraging and reassuring me I could accomplish this degree. I would like to especially thank Ryan Bartels, my husband for all of his support and words of wisdom in times when I thought I could never finish. To you all I am forever grateful.

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CHAPTER 1

Introduction

The family *Potyviridae* comprises more than 40% of the known plant viruses with eight different genera, making it one of the most economically important plant virus families (Shukla *et al.,* 1994; Betty *et al.,* 2008; Adams *et al.,* 2011). The *Potyviridae* family is composed of (+)-sense, single-stranded RNA viruses with mono- or bi-partite viruses. Their genome is translated as a single polyprotein that is cleaved into at least 10 mature proteins. An abundant amount of research has been done to understand these RNA viruses, with the goal of controlling their impact on agronomically important crops.

Several functions of potyviral proteins have been determined for their role in the virus disease cycle. Protein 1 (P1) functions as a proteinase that cleaves between itself and helper component-proteinase (HC-Pro) and is required for efficient genome amplification (Verchot and Carrington, 1995; Choi *et al.,* 2002). The P1 proteins of *Triticum mosaic virus* (TriMV), *Sugarcane streak mosaic virus* and *Wheat streak mosaic virus* (WSMV) are responsible for suppressing host RNA silencing (Young *et al.,* 2012; Tatineni *et al.,* 2012). In contrast, HC-Pro functions as a suppressor of RNA silencing within species of the *Potyvirus* and *Rymovirus* genera (Anandalakshmi *et al.,* 1998; Kasschau and Carrington, 1998; Young et al., 2012). HC-Pro plays a major role in effective transmission of the virus to the host plant by their arthropod vectors (Atreya *et al.,* 1992; Stenger *et al.,* 2006) and functions as a *cis*-acting protease that processes the polyprotein (Carrington *et al.,* 1989; Oh and Carrington, 1989). Additionally, HC-Pro appears to be involved in several other stages of the virus disease cycle, including disease synergism (Pruss *et al.,* 1997; Shi *et al.,* 1997), replication (Kasschau and Carrington, 1995; Klein *et al.,* 1994; Kasschau *et al.,* 1997), cell-to-cell and long-distance movement (Atrey and Pirone, 1993). The P3 protein is a key determinant of pathogenicity and

virulence in several potyviruses (Sáenz *et al.,* 2000; Johansen *et al.,* 2001). P3 contains a conserved region known as PIPO which function in cell-to-cell movement of the virus (Wen and Hajimorad, 2010; Wei *et al.,* 2010). Cylindrical inclusion body protein (CI) has ribonucleotide hydrolysis and RNA helicase activities which are essential for virus RNA replication (Fernandez *et al.,* 1995, 1997; Lain *et al.,* 1990, 1991). In addition, CI is critical for both cell-to-cell and long-distance movement within the host plant (Langenberg, 1993; Carrington *et al.,* 1998). The 6K1 and 6K2 proteins are short proteins and their functions are unknown. The third virus-encoded proteinase is nuclear inclusion protein a (NIa) which helps cleave the polyprotein (García *et al.,* 1990). NIa serves as the VPg, a viral protein covalently bound to the 5'-terminus of genomic RNA (Shahabuddin *et al.,* 1988; Murphy *et al.,* 1990). Nuclear inclusion protein b (NIb) is assumed to be the viral RNA-dependent RNA polymerase (RdRp) because of the presence of conserved polymerase motifs (Domier *et al.,* 1986; Lain *et al.,* 1989). The coat protein (CP), a structural protein, is also required for aphid transmission (Atreya *et al.,* 1991; Gal-On *et al.,* 1992) and is essential for both cell-to-cell and long-distance movement within infected plants (Dolja *et al.,* 1995; Rojas *et al.,* 1997; Tatineni *et al.,* 2014). These proteins are encoded by the genomes of the majority of potyviruses functioning in viral virulence and the disease cycle.

Triticum mosaic virus (TriMV) was first reported in 2006 in Kansas from wheat (*Triticum aestivum L.*) plants displaying mosaic symptoms (Wolf and Seifers, 2008; Seifers *et al.,* 2008). TriMV has also been identified in wheat in the Great Plains region states of Nebraska, Oklahoma, South Dakota, Wyoming, Texas and Colorado, and more recently, North Dakota and Montana in 2009 (Seifers *et al.,* 2011). The specific amount

of grain yield loss annually to TriMV has yet to be established. However, fields infected with TriMV do display various yield losses: from 1% up to 76% depending on the wheat cultivar (Seifers *et al.,* 2011; Byamukama *et al.,* 2014). TriMV host range includes wheat, barley, rye, oat, triticale and certain wild grass species (Seifers *et al.,* 2010; Tatineni *et al.,* 2010).

TriMV is the type species of the genus *Poacevirus* in the family *Potyviridae* (Tatineni *et al.,* 2009). TriMV mature proteins display a relatively high amino acid identity of 47% to 64.8%, to *Sugarcane streak mosaic virus* (SCSMV) mature proteins (Tatineni *et al.,* 2009; Fellers *et al.,* 2009). When comparing sequences of TriMV to WSMV, a sequence identify of 17% to 41% was observed (Tatineni *et al.,* 2009). Particles of TriMV are flexuous rods, approximately 15 nanometers (nm) in diameter and 800 nm in length (Seifers *et al.,* 2008). TriMV is a (+)-sense, single-stranded RNA virus, consisting of 10,266 nucleotides (nt), not including the poly A tail (Fellers *et al.,* 2009; Tatineni *et al.,* 2009). The genomic RNA is translated as a polyprotein of 3,112 amino acids that is cleaved into at least 10 mature proteins when processed by three viral proteinases (P1, HC-Pro and NIa).

TriMV infected wheat plants display mosaic, chlorotic spotting, or mottling symptoms that are indistinguishable from those of WSMV (Wolf and Seifers, 2008). If wheat plants are infected at an early growth stage, severe stunting, reduced tillering, poor seed set and lower seed weight can be observed. In susceptible wheat cultivars, co-infection by TriMV and WSMV, result in synergistic interaction with increased virus accumulation and symptom severity (Tatineni *et al.,* 2010; Byamukama *et al.,* 2013, 2014). Co-infection of WSMV and TriMV in susceptible wheat cultivars may cause

death of infected plants or cause severe yield loss up to 96% depending on the wheat cultivar (Tatineni *et al.*, 2010; Byamukama *et al.*, 2013, 2014).

TriMV is transmitted by the wheat curl mite (WCM, *Aceria tosichella*) in a similar manner to that of WSMV (Slyhkuis, 1955; Seifers *et al.*, 2009; McMechan *et al.*, 2014). However, the nature of mite transmission of TriMV and WSMV has not been clearly established. Wheat curl mites are wingless and depend entirely on wind for their dispersal. Green living plant tissue is necessary for mite survival. Without a food source they will perish within days. TriMV acquisition and inoculation feeding time, for wheat curl mites are currently unknown.

It is speculated the disease cycle of TriMV is similar or identical to that of WSMV. The disease cycle begins with the presence of over-summering hosts (grasses and volunteer wheat) that allow wheat curl mites to survive and reproduce over the summer. The over-summering hosts permit the transmission of TriMV from the previous season's infected winter wheat crop to be carried over to the next season's crop. The mites are able to increase their population throughout the summer and transmit the virus to the newly emerging wheat in the fall. In the Great Plains region, wheat that emerges before harvest of the summer host creates a "green bridge" for the mites. This permits the mites to shift from their over-summering host to volunteer wheat or alternative hosts (green foxtail, barnyardgrass, or jointed goatgrass) and survive until the new wheat crop begins to sprout (Wegulo *et al.*, 2008). Once winter wheat begins to emerge, the mites can transmit TriMV to the seedlings, here the virus will survive over the winter. For this reason, it is import to control volunteer wheat in regions where TriMV is a problem. The

disease cycle can be broken by removing volunteer wheat and targeting the "green bridge".

Viral RNA populations are comprised of variant genomes due to the high error rate of their viral polymerase, large population sizes, and short replication cycles. RNA-dependent RNA polymerases (RdRp) have an error rate of approximately 10^{-4} per nucleotide per replication, and lack proof-reading capabilities (Hall *et al.,* 2001; Harrison, 2002). This high error rate permits RNA viruses to increase their genetic diversity and undergo rapid evolutionary changes (Novella *et al.,* 1995). Within individual RNA virus isolates low levels of variation have been found (Albiach-Martí, *et al*., 2000; Kong *et al.,* 2000; French and Stenger, 2003; García-Arenal *et al.,* 2003). However, high sequence diversity between plant RNA virus isolates has been observed (Kurath and Palukaitis, 1990; French and Stenger, 2003; García-Arenal *et al.,* 2003). Therefore, evolutionary and population genetics factors, such as purifying selection and/or episodes of genetic bottlenecks favoring genetic stability, must play a role for many plant viruses (Rodriguez-Cerezo *et al.,* 1991; Hall *et al.,* 2001; Schneider and Roossinck 2001; Li and Roossinck *et al.,* 2004; French and Stenger, 2005; Acosta-Leal *et al.,* 2011).

It is imperative to understand how virus populations evolve over time in order to develop effective management strategies. Changes within a viral population may cause an emergence of severe isolates. Several studies have addressed how a mutation of a single amino acid can affect virus virulence. For example, a single amino acid alteration within the HC-Pro of WSMV restored long distant movement capabilities in a systemically defective virus (Bartels, 2011). For *Turnip crinkle virus*, a member of the *Tombusviridae* family, a single amino acid substitution within its movement protein

rendered it avirulent (Wobbe *et al.,* 1998). One amino acid change in *Cucumber mosaic virus* RNA polymerase determines whether it has a virulent or avirulent phenotype on cowpeas (Karasawa *et al.,* 1999). These examples are just a few displaying the importance of understanding the dynamics of virus populations.

Recent research with a limited number of field collected TriMV isolates has found that populations of TriMV in the Great Plains region are homogenous with little variation (Fuentes-Bueno *et al.,* 2011; Seifers *et al.,* 2013). To assess the TriMV population dynamics, the following research questions were examined: Is variation within TriMV populations similar to that of WSMV, another wheat-infecting virus? Is the variation in the CP cistron driven by host specificity? Lastly, is genetic stability of the TriMV population maintained when TriMV is passed from wheat to an alternative host back to wheat using its natural vector the wheat curl mite? To address the first question, a Nebraska isolate of TriMV was serially passed through winter wheat and analyzed by single-strand conformational polymorphism (SSCP) for variability within the P1 and CP cistron. To assess the second question, two TriMV isolates with a single nucleotide substitution in the CP cistron were mechanically inoculated onto four host species and analyzed by SSCP for genetic variation. To evaluate the third objective, a Nebraska isolate of TriMV was mechanically inoculated onto wheat plants and infested with wheat curl mites. The mites were then transferred to alternative hosts and then back to wheat prior to analyzing the P1 and CP cistrons using SSCP to determine genetic variation.

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CHAPTER 2

Population Variation in *Triticum Mosaic Virus* Serially Passaged in Winter Wheat

ABSTRACT

A series of serial passages of *Triticum mosaic virus* (TriMV) was performed in winter wheat under greenhouse conditions to examine the dynamics of sequence variation in the TriMV population. The variation within the protein 1 (P1) and the coat protein (CP) of TriMV was assessed by single-strand conformational polymorphism (SSCP) assay, followed by nucleotide sequencing. Three lineages were established to compare diversity between and within each TriMV population. Different SSCP patterns among clones were associated with single-nucleotide substitutions. The new haplotypes detected within the P1 and CP cistrons were mostly random, with little correlation between lineages or passages. These results are consistent with stochastic changes due to genetic drift which have been reported to occur in population structures after repetitive and severe bottlenecks. TriMV mutations observed in the populations after serial passages were dominated by singletons within the P1 and CP cistrons, with no obvious bias to particular regions. Examination of non-consensus variation revealed the number of unique substitutions remained constant in frequency during passaging. These results suggest that stochastic processes such as bottlenecking are impacting the population. The mutation frequency per nucleotide was discovered to be 2.231×10^{-4}/nt at passage 9 within the CP cistron. The mutation frequency of the P1 cistron was determined to be 0.580×10^{-4}/nt. The mutation frequencies determined were low, not only compared to other *Potyviridae* family members, but also compared to other (+)-sense, single stranded plant RNA viruses in general. It was concluded that negative selection and genetic drift must be evolutionary processes influencing TriMV populations.

INTRODUCTION

RNA viruses have a high mutation rate, short replication time, and a particularly large population size (McNeil *et al.*, 1996; Hall *et al*., 2001; Harrison, 2002; French and Stanger, 2003; García-Arenal *et al.*, 2003; Elena *et al.*, 2008; Gao and Feldman, 2009). The high mutation rate is due to the high error rate of RNA-dependent RNA polymerase, estimated at 10^{-4} per nucleotide per replication, a consequence of a lack of proof-reading capability (Drake, 1993; Hall *et al*., 2001; Malpica *et al.*, 2002; Harrison, 2002; García-Arenal *et al.*, 2003; French and Stenger, 2003, 2005). This permits RNA viruses to undergo rapid evolutionary changes and increase their genetic diversity (Novella *et al.*, 1995; Hall *et al.*, 2001). However, when sequence diversity in plant RNA viruses has been studied within individual isolates, relatively low levels of variation have been found (Kurath and Palukaitis, 1990; Rodriguez-Cerezo *et al.*, 1991; Albiach-Martí, *et al*., 2000; Kong *et al.*, 2000; French and Stenger, 2003; García-Arenal *et al.*, 2003). This suggest that for many plant viruses, evolutionary factors, such as genetic bottlenecking and negative selection, must play a role in favoring genetic stability (Rodriguez-Cerezo *et al.*, 1991; Hall *et al.*, 2001; García-Arenal *et al.*, 2003; Li and Roossinck, 2004; French and Stenger, 2005, Acosta-Leal *et al.*, 2011).

Viruses must travel through plasmodesmata linking plant cells in order for systemic movement to occur. This forces them through severe bottlenecking episodes (Lucas and Gilbertson 1994; Carrington *et al.*, 1996, 1999; Hall *et al.*, 2001; French and Stenger, 2003; Li and Roossinck, 2004; French and Stenger, 2005). This restriction due to virus movement causes severe bottlenecking events, affecting the overall population

dynamics (Hall *et al.*, 2001; French and Stenger, 2003, 2005; Sacristán *et al*., 2003; Li and Roossinck, 2004; Elena *et al.,* 2008; Monsion *et al.,* 2008). The multiplicity-of-infection (MOI) of a plant virus is known as the number of virus genomes invading an adjacent non-infected cell to initiate an infection. The MOI of a plant virus can predict the severity of bottlenecking events. Consequently, MOI plays a role in virus evolution, selection intensity on genes, genetic exchange, epistatic interactions, hybrid incompatibility, hyperparasitism and the evolution of multipartite and segmented genomes (González-Jara *et al.,* 2009; Miyashita and Kishino, 2010; Gutiérrez *et al.,* 2010; Bartels, 2011). A high MOI favors genetic diversity within the virus population, but slows down virus evolution by decreasing selection intensity. The relatively low MOI determined for several plant viruses, ranging from 5 to 11 functional genomes of the approximate 10 million total genomes per cell, causes an increase in viral evolution via random genetic drift, but a reduction in genetic diversity within the virus population as a whole (Sacristán *et al*., 2003; González-Jara *et al.,* 2009; Miyashita and Kishino, 2010; Gutiérrez *et al.,* 2010; Bartels, 2011).

Triticum mosaic virus (TriMV) is the type member of the genus *Poacevirus* in the family *Potyviridae* (Seifers *et al.,* 2008; Fellers *et al.,* 2009; Tatineni *et al.,* 2009; Adams *et al.,* 2011). TriMV is a (+)-sense, single-stranded RNA virus, consisting of 10,266 nucleotides excluding the poly A tail (Fellers *et al.,* 2009; Tatineni *et al*., 2009). The genome is translated into a single polyprotein that is cleaved into at least 10 mature proteins by three putative viral proteinases (P1, HC-Pro and NIa) (Fellers *et al.,* 2009; Tatineni *et al*., 2009). TriMV is vectored by the wheat curl mite (*Aceria tosichella*), another potential source of bottlenecking for the population (Seifers *et al.,* 2009). Studies

with a limited number of TriMV isolates from field samples within the Great Plains region were found to be homogenous, with little sequence variation (Fuentes-Bueno *et al.,* 2011; Seifers *et al.,* 2013).

A Nebraska isolate of TriMV (Tatineni *et al.,* 2009) was serially passaged to evaluate the possible occurrence of variation over time. The experiment utilized the single-strand conformation polymorphism assay (SSCP) to identify differences in lineages at various time points from the initial parental sequence. SSCP is a useful tool utilized to identify single nucleotide variations within genes in virus populations (Orita *et al.,* 1989; Rubio *et al,* 1996; Welsh *et al.,* 1997; Pawlotsky *et al.,* 1998; Hall *et al.,* 2001; Kong *et al.,* 2003; Gasser *et al.,* 2006; Goszczynski, 2007). SSCP analysis relies on the bulk property of PCR, permitting the predominant sequence in the reactions to be measured. The electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is highly dependent on its size and structure, thus the basis of SSCP (Orita *et al.,* 1989; Gasser *et al.,* 2006). This approach allowed a large number of samples to be screened for genetic variation without incurring the cost of sequencing.

This approach allowed us to confidently sample the population dynamics of TriMV within three lineages during serial passages. SSCP permitted polymorphisms to be identified in the protein 1 (P1) and coat protein (CP) regions of TriMV across multiple lineages. P1 is reported to be the least conserved domain in monopartite potyviruses (Valli *et al.,* 2007; Seifers *et al.,* 2013). Conversely, the CP region is known to be highly conserved, and research has found this to be the case for TriMV (Fuentes-Bueno *et al.,* 2011; Seifers *et al.,* 2013). This research permitted us to determine the base mutation frequency per nucleotide of TriMV for the P1 and CP cistrons.

MATERIALS AND METHODS

Preparation of TriMV plasmid

A TriMV cDNA infectious clone was created in pUC118 using a Nebraska isolate of TriMV, recovered near Red Willow County, Nebraska (GenBank, accession no. FJ669487) (Tatineni *et al.,* 2009; Tatineni, unpublished). pTriMV was transformed into *E. coli* strain JM109 competent cells (Promega, Madison, WI). Plasmid DNA was then isolated from 40 ml cultures grown overnight using Perfectprep Plasmid Midi Kit (BioRad, New England) as per the manufacturer's protocol.

The isolated plasmid DNA was verified by restriction enzyme digestion with *Pst* I (Promega, Madison, WI) and *Nco* I (BioLabs, New England) for one hour at 37˚C, followed by agarose gel electrophoresis. pTriMV was linearized by digesting with *Not* I restriction enzyme (BioLabs, New England) at 37˚C for two hours, followed by phenol:chloroform extractions and ethanol precipitation. Linearized pTriMV DNA was resuspended in 60 µl sterile water and DNA concentration was measured using a Nano-spectrophotometer (IMPlen). The DNA was stored frozen at -20˚C.

Inoculation of passages

The SP6 MEGAscript, High Yield Transcription Kit (Ambion, Austin, TX) was used to generate *in vitro* transcripts using *Not* I-linearized pTriMV as the template as per manufacturer instructions. Three lineages of TriMV were established, lineages A, B, and C. For lineage A, B, and C, at each passage wheat (*Triticum aestivum* L. cv. Tomahawk) seeds were planted in a six-inch diameter clay pot in a pre-mixed sterilized potting

medium. To minimize differential selective pressures due to environmental conditions, all lineages were maintained in a 20˚C to 26˚C greenhouse and watered daily.

For the first passage, three pots of seven day old wheat seedling at the two- leaf stage were inoculated with *in vitro* transcripts of TriMV. One pot of wheat seedlings was planted as a control and was inoculated with buffer. For serial passages of TriMV, the three separate lineages were maintained for 24 serial passages performed at 14 day intervals. A single passage in one lineage consisted of inoculating 16 to 18 wheat seedlings at the two-leaf stage within a common pot. For each lineage at each passage, one systemically infected symptomatic leaf was collected from the corresponding lineage at 14 days post inoculation (dpi) and mechanically inoculated onto new wheat seedlings at the two-leaf stage. A crude plant sap extract diluted 10^{-1} in sterile water was used for inoculation of wheat seedlings at the two-leaf stage in all serial passages.

Total RNA extraction

At 14 dpi, two upper systemic leaves were collected from lineages A, B, and C, at every third passage starting with passage nine until passage 32. Viral RNA was extracted from harvested systemic leaves as follows. Samples were placed in a grinding bag with 1 ml of sterile water and hand ground until the leaves became slightly translucent. For each sample, 250 µl of leaf extract was transferred to a fresh 1.5 ml tube containing 250 µl of 2x glycine buffer (0.2 M Glycine, 20 mM EDTA, 0.2 M NaCl, pH 9.0), 25 µl of 20% SDS and 500 µl of phenol:chloroform:isoamyl alcohol. The samples were vortexed thoroughly and centrifuged at 13,200 rpm for five minutes. Approximately, 300 µl of the aqueous phase was transferred to a fresh 1.5 ml tube on ice, where 30 µl of 3M sodium

acetate (pH 4.8) and 900 µl of 100 % ethanol was added and inverted. Samples were centrifuged at 13,200 rpm for five minutes at 4ºC. Pellets were washed with 500 µl of 70% ethanol, inverted and centrifuged at 13,200 rpm for three minutes, then vacuum dried and resuspended in 25 µl of sterile water. The extracted RNA was stored at -80˚C.

Reverse transcription (RT) and polymerase chain reaction (PCR)

RT-PCR was performed on RNA extracted from each lineage/passage using P1 and CP specific primers. Primers targeting part of the 5'UTR and whole P1 region of TriMV consisted of a forward primer annealing to nucleotide position 301 to 318, (Tr-72 5'-GATGAGCTCTACAAATAAGGGCTTAGGCGATTGTAC-3') and a reverse primer annealing to nucleotide position 1,860 to 1,888, (Tr-76 5'-GAGAGAGCTCCTAGTAATATGTCAAGCCCTCTAAGCAATCAG -3'). Primers annealing to the CP region of TriMV were comprised of a forward primer annealing to nucleotide position 9,001 to 9,019, (Tr-89 5'-CAAGATTAACGCGGCATGG-3') and a reverse primer annealing to nucleotide position 10,048 to 10,075, (Tr-100 5'-AACCTCGAGCTAACGGGTACCAAACATGGCCCCGCCGACA-3').

Reverse transcription was performed on viral RNA samples at 42˚C for 1 hour, followed by 99˚C for 5 min and held at 10˚C. Each reverse transcription reaction consisted of 5.9 µl of sterile water, 2 µl of 5x buffer (RT AMV), 0.5 µl of 50 ng/µl virus specific reverse primer, 0.4 µl of 10 mM dNTPs, 0.2 µl of RT AMV (Roche, Indianapolis, IN) (23 U/µl) and 1 µl of total RNA isolated from upper systemic leaves. Two PCR reactions were performed on each lineage sample for passages 9, 18, and 24 targeting the P1 or CP regions using high fidelity *Pfu* II Ultra polymerase by Agilent

(Figure II-1). One µl cDNA was used for PCR in a 25 µl reaction volume with the P1 or CP specific primers. The PCR program consisted of: 95˚C for 2 min, followed by 10 cycles at 95°C for 30 s, 48˚C for 30 s and 72˚C for 2 min. The annealing temperature was increased to 56˚C for the remaining 30 cycles, for a total of 40 cycles on a T1 thermocycler (Biometra). All reactions were held at 25˚C until removed from the thermocycler and stored at -20˚C. The RT-PCR products were analyzed through 1.0% agarose gels in TBE (Tris-borate-EDTA) buffer.

Cloning of RT-PCR products

A Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Life Technologies) was used to clone P1 and CP PCR products into One Shot chemically competent *E. coli*. The manufacturer's protocols were followed as written. Twenty clones were selected from each transformation for inoculation of minipreps. A Fermentas Life Science GeneJet Plasmid Miniprep Kit (Pittsburgh, PA) was utilized to isolate the plasmids from the minipreps, following the supplied protocol. Plasmids containing the insert were digested with restriction enzyme *Eco*RI (BioLabs, New England) at 37˚C to release the PCR fragment. Digested plasmids were diluted to 1:500 with sterile water, and re-amplified by PCR using the previously described reaction mixture with the appropriate primer set and program.

Single-strand conformation polymorphism (SSCP) analysis

SSCP analysis was performed on 20 positive clones from passage 9, 18, and 24 from each lineage (A, B, and C) for both the P1 and CP regions (Figure II-1). The final

PCR products were digested with appropriate restriction enzymes to digest into ~200 to 500 bp fragments required for SSCP analysis (Figure II-2) (Orita *et al.,* 1989). Three restriction enzymes, *Ban* I, *Pst* I and *Pvu* II were utilized to digest the P1 PCR products of each clone into four fragments. Restriction enzyme *Dde* I digested the CP PCR product of each clone into four fragments. Digested samples were prepared for SSCP as described in Hall *et al.,* (2001). Ten percent Tris/Borate/EDTA (TBE) polyacrylamide gels (Life Technologies, Carlsbad, CA) were electrophoresed in 1X TBE buffer at 20ºC and 4ºC at 60 volts for 16 ½ hours and 45 volts for 15 ½ hours, respectively. The accuracy of SSCP to detect single nucleotide polymorphisms improves to ~98% when two gels are electrophoresed, each at alternate temperatures (Welsh *et al.,* 1997). Silver staining of SSCP gels was performed using a GE Healthcare DNA Silver Staining Kit PlusOne (Waukesha, WI), and following the protocol provided (Pawlotsky *et al.,* 1998). Samples displaying SSCP patterns dissimilar from the positive control, (plasmid TriMV), were prepared for sequencing.

Plasmids prepared for sequencing

The miniprep DNAs (30 µl) were resuspended in 170 µl of Tris-EDTA buffer (pH 8) and treated with 2 µl RNase A (1ng/µl) for 20 minutes at 37ºC, and then extracted with phenol:chloroform as follows: sterile ultra-purified water was added to each sample to bring the volume to 300 µl, followed by adding 300 µl of phenol:chloroform and vortexed thoroughly, centrifuged at 13,200 rpm for four minutes. The aqueous phase was collected and 400 µl of chloroform:isoamyl alcohol (24:1) was added to each sample. The sample was mixed thoroughly, and centrifuged at 13,200 rpm for four minutes and

then the aqueous phase was collected and 1/10 the volume (~25 µl) of 3M sodium acetate (pH 4.8) and 2.5 volume (~625 ml) of 100% ethanol were added. Samples were incubated for 15 minutes at -80°C, and then centrifuged at 13,200 rpm for 15 minutes. Supernatant was decanted, and the pellets were washed with 500 µl of 70% ethanol, vortexed and then centrifuged at 13,200 rpm for ten minutes. Supernatant was decanted and pellet was vacuum dried. Total RNA was resuspended in 25 µl water. Samples were sequenced at the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida. Sequence results were analyzed by Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI).

Error rate of PCR

To determine the intrinsic error rate of the PCR system, the TriMV plasmid used to make transcripts for the first passage inoculation was amplified by PCR using the P1 and CP specific primers. The PCR products were digested and cloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Life Technologies) as described above. Fourteen clones for both the P1 and CP were screened for polymorphisms and sequenced. All mutations detected were attributed to *Pfu* II Ultra polymerase errors introduced during PCR.

Statistical Analysis

Statistical analysis was completed using SAS version 9.2 (SAS Institute Inc.). Two-way Analysis of Variance (ANVOA) and Chi-Square tests were used to address differences within and between passages, lineages, and/or proteins.

RESULTS

Haplotypes found by SSCP in the P1 cistron

The TriMV P1 cistron data set consisted of 51,986 sequenced nucleotides with a total of 275,220 nucleotides screened for polymorphisms by SSCP (Figure II-1). SSCP analysis was used to assess the population dynamics of TriMV to be sampled at three time points: passage 9, 18, and 24. Haplotypes were identified by variation in SSCP patterns as illustrated in Figure II-2 and summarized in Table II-1. For P1 and CP, the infectious clone sequence was designated haplotype A.

The prevalence of the founding haplotype A was maintained in the P1 samples (Table II-1). Lineage A preserved haplotype A frequency across passages 9 and 18 with 18 clones; however, this value decreased slightly to 16 clones by passage 24 (Figure II-3) ($P \geq 0.8642$). No significant difference in the frequency of haplotype A was detected among passages 9, 18, and 24 for lineage B with 15, 16, and 19 clones, respectively ($P \geq 0.6076$). A significant increase in the number of haplotype A clones was detected in lineage C. Comparing passage 9 of lineage C with no haplotype A clones to passage 18 with 14 or passage 24 with 19, provided a significant *P* value of 0.0001 and 0.000004, respectively. Lineages A, B, and C of P1 were also compared by passage. At passage 9, lineage C was significantly different in the number of haplotype A clones compared to lineages A and B ($P = 0.000008$ and $P = 0.00006$). No significant differences were detected at passages 18 or 24 between lineages A, B, and/or C.

By combining lineages A, B, and C together, a better representation of haplotype A frequency within a repeatedly passaged TriMV population was obtained (Figure II-4).

At passage 9, 33 of the 60 clones evaluated by SSCP for polymorphisms within P1 were haplotype A, leaving 27 as variants. At passage 18, this figure increased to 48 of the TriMV population representing haplotype A and 12 as unique haplotypes. Analysis of passage 24, revealed that nearly a complete reversion of the population to the founding haplotype A had occurred, with 54 of the clones identified as haplotype A and six as single distinct haplotypes.

Haplotypes found by SSCP in the CP cistron

The CP cistron data sets consisted of 47,235 sequenced nucleotides, with total of 180,900 nucleotides screened for polymorphisms by SSCP. CP haplotypes diverged away from the founding haplotype A as passages increased (Table II-1). For lineage A, the frequency of haplotype A was maintained across passages 9 and 18, at 16 and 15 clones with a slight increase to 18 clones by passage 24 (Figure II-5) ($P \geq 0.7283$). A significant shift from haplotype A to variant haplotypes was seen in lineage B with 18 clones in passage 9, decreasing to zero clones in passages 18 and 24 ($P = 0.000008$). A similar trend was observed across passages within lineage C, with haplotype A accounting for 13, zero, and zero clones. Accordingly, a significant decrease in the frequency of haplotype A was observed in lineage C when comparing passage 9 to passages 18 or 24 ($P = 0.0002$).

Comparing the number of haplotype A clones within each lineage of CP by passages, significant differences between lineages were identified. Lineages A, B, and C were not significantly different at passage 9 ($P \geq 0.4731$). At passage 18, lineage A had significantly more haplotype A clones compared to lineages B or C ($P = 0.00006$). The

occurrence of haplotype A at passage 24 was significantly different when comparing lineage A to lineage B or C (P = 0.000008). By merging lineages A, B, and C and treating them as replications per passage, a better assessment of haplotype A frequency within a repeatedly passaged TriMV population was achieved (Figure II-4). At passage 9, 47 of the 60 CP clones examined by SSCP were identified as haplotype A, the remaining 13 were diverse haplotypes (Figure II-4). The number of haplotype A clones within the overall TriMV population dropped to 15 by passage 18, meaning 45 of the clones were variant haplotypes. By passage 24, the founding haplotype A was identified in 18 of the clones, with 42 being different haplotypes (Figure II-4).

A significant difference in haplotype A occurrence over the three passages was detected between TriMV P1 and CP (P = 0.0458). Closer examination revealed that the frequency of haplotype A was not significantly different at passage 9 between the two cistrons (P = 0.3385). Although, by passage 18, a significant difference was detected between P1 and CP in the frequency of haplotype A (P = 0.0393). The deviation continued into passage 24 between the two cistrons, with a significant P value of 0.0272.

Haplotype consensus found in P1 or CP

Nucleotide substitution consensus among P1 and CP haplotypes is illustrated in Table II-2, with the haplotypes presented having appeared multiple times within the data sets. The substitutions which comprise haplotype I, possibly appeared early in the viral passages because it was identified in 17 of 20 clones sampled at passage 9, lineage C (Table II-2). Haplotype I consisted of three mutations with two inserts, a U at nucleotide position 726 and a C at position 727 in the 5'UTR, one non-synonymous mutation, G_{850}

to T, caused an amino acid change from lysine to asparagine. Two samples in passage 24, lineage A exhibited haplotype Z, which consisted of one synonymous substitution of a U for a C in the codon of histidine at nucleotide position 1,564.

The four haplotypes shown in Table II-2 were identified more than once for the CP cistron. Haplotype t, which contained a synonymous substitution of G to A at position 9,478, was seen a total of 57 times within passage 18, lineage B and C, and preserved into passage 24 for both lineages (Table II-1). Two synonymous substitutions within the leucine (G_{9478} to A) and serine (C_{9790} to U) codons were characterized as haplotype y and discovered twice in passage 18, lineage C. Four clones from passage 24, lineage B represented haplotype cc, consisting of two synonymous mutations in the amino acid codons of leucine (G to A) at position 9,478 and aspartic acid (C to U) at position 9,550. Haplotype ee was identified in eight clones in passage 24, lineage B and contained one synonymous mutation of leucine (G_{9478} to A) and one non-synonymous mutation of C_{9591} to U causing a shift from alanine to valine.

Substitution analysis of polymorphic sites within the P1 cistron

Several polymorphisms were discovered within part of the 5'UTR and the P1 cistron by SSCP and verified by sequencing. Substitution occurred throughout the cistron with no obvious bias to particular regions. A summary of the 39 singleton and five non-singleton substitutions detected are presented in Table II-3. Singleton mutations are those that occur once within the data set; conversely, non-singletons are substitutions which arise multiple times. Insertions within the 5'UTR were detected numerous times

within the data set however; this would not disrupt translation of the open reading frame sequence.

Lineage A had 12 substitutions detected over the three passages, four were non-synonymous, six were synonymous and two were located in the 5'UTR (Table II-3). In passage 24 of lineage A, a synonymous substitution of U_{1564} for C within the histidine codon was detected in four clones. A total of 15 unique mutations were discovered over the three time points within lineage B, five were within the 5'UTR, eight were non-synonymous and two were synonymous substitutions (Table II-3). The largest number of mutations was detected in lineage C with 77. Of those, 46 occurred within the 5'UTR, 30 were non-synonymous mutations and one synonymous mutation. For passage 9, lineage C, all 20 clones revealed inserts of U and C, at positions 726 and 727 within the 5'UTR, respectively. In addition, a non-synonymous mutation $G_{850} \rightarrow U$ caused an amino acid change from lysine to asparagine in all clones from passage 9, lineage C (Table II-3). These substitutions may have arisen early in the infection process to occur at a high enough frequency to be identified within every clone sampled. In lineage C passage 18, a substitution of U for A at nucleotide position 1,421 created a stop codon which would mostly likely result in a defective virus (Table II-3).

Substitution analysis of polymorphic sites within the CP cistron

Within the CP cistron, nine non-singleton and 32 singleton substitutions were discovered and are presented in Table II-4. Substitutions arose throughout the entire cistron with no apparent regional bias. Lineage A across passages 9, 18, and 24 had 21 substitutions detected within the CP region, of those 17 were non-synonymous and four

were synonymous. A non-synonymous mutation at nucleotide position 9,423 altered lysine to arginine and was maintained within lineage A from passages 9 through 24 (Table II-4). This mutation possibly became fixed before passage 9 sampling, since it was detected within all clones sequenced for lineage A. Substitution of $C_{9917} \rightarrow U$ in passage 9, lineage A resulted in a stop codon instead of the amino acid arginine, producing a defective or lethal mutation in the genome.

Across the three passages, 63 mutations were discovered within lineage B, 47 were synonymous and 16 were found to be non-synonymous (Table II-4). Two deletions were detected in lineage B, passage 18 at nucleotide positions 9817 and 9818; this would cause a two-nucleotide frame shift, possibly resulting in defective or lethal mutations in the virus genome (Table II-4). A synonymous mutation of $G_{9478} \rightarrow A$ in the leucine codon was identified in each clone sampled from lineage B and C, in passage 18 and 24 (Table II-4). Within lineage C, 54 substitutions were observed with 42 being synonymous and 12 being unique non-synonymous. All other substitutions observed within the CP region for each lineage occurred merely once within the screened data set.

Error rate of reverse transcriptase and PCR

The error rate of the RT-PCR system employed for this research was assessed. The TriMV infectious clone used for the inoculation of passage 1 was processed and prepared in the same manner as the passaged samples. Mutations identified within this data set were attributed to polymerase errors introduced during PCR. For the P1 cistron, when 14 clones were screened for polymorphisms and verified by sequencing; only one was found (Table II-5). These results permitted a calculated error rate of *Pfu* II Ultra

DNA polymerase used in amplifying the P1 cistron at 0.46716 x 10^{-4}/nt. No polymorphisms were revealed within the 14 clones of the CP region (Table II-5). The *Avian Myeloblastosis Virus* (AMV) reverse transcriptase (RT) error rate has been previously identified as one error per 17,000 nucleotides transcribed (0.588 x 10^{-4}/nt) (Roberts *et al.*, 1988; Roberts *et al.*, 1989; Menéndez-Arias 2009). The RT-PCR error rate was calculated by adding the error rate for RT and the error for *Pfu* II Ultra DNA polymerase (Table II-5). The RT-PCR error rate for the P1 cistron was calculated as 1.055 x 10^{-4}/nt and for the CP cistron the error rate was 0.588 x 10^{-4}/nt.

Mutation rate of TriMV within the P1 and CP cistron

The base mutation frequency per nucleotide within TriMV P1 and CP cistrons was identified. Lineages A, B, and C for each passage were treated as replications for calculating the base mutation frequency per passage for each cistron. For mutations arising multiple times within a lineage/passage combination, that mutation was counted once for the number of polymorphisms observed, in order to calculate the mutation frequency (Table II-3 and II-4). The mutation frequency observed in the P1 cistron by passage 9 gave a corrected mutation frequency of 0.580 x 10^{-4}/nt (Table II-6). By passage 18, the mutation frequency increased to 1.234 x 10^{-4}/nt. The mutation frequency could not be determined from the given data for passage 24 due to the RT-PCR error, which was greater than the observed mutation frequency. Consequently, it cannot be distinguished whether the eight polymorphisms seen in passage 24 were true polymorphism or artifacts of RT-PCR. The mutation frequency of P1 within passage 9 compared to passage 18 gave a *P* value of 0.3517, this value increased to 0.8439 when

comparing passage 18 to 24. Both values suggest there was no significant difference in the mutation frequencies observed across passages within the P1 region. For the CP cistron, a higher mutation frequency of 2.231 x 10^{-4}/nt was revealed at passage 9 compared to the P1 cistron (Table II-6). The mutation frequency obtained at passage 18 was 2.065 x 10^{-4}/nt. Comparing the mutation frequency of passage 9 to that of passage 18 for the CP cistron, no significant difference was identified (P = 0.1676). At passage 24, the mutation frequency was determined to be 1.236 x 10^{-4}/nt. No significant difference was found when comparing the mutation frequency of passage 18 to that of passage 24 (P = 0.9675).

Statistically comparing the mutation frequencies obtained from TriMV P1 and CP by each passage revealed low P values of 0.1357 and 0.0883 at passage 18 and 24, respectively. The mutation frequency between P1 and CP at passage 9 gave a P value of 0.4134. The mutation frequency per nucleotide for a single passage was determined by dividing each corrected mutation frequency per nucleotide by the number of passages away from passage 1 (the starting homogenous inoculum). The P1 cistron has a relatively low mutation frequency per nucleotide for a single passage of 0.064 x 10^{-4}/nt to 0.069 x 10^{-4}/nt and the mutation frequency per nucleotide of CP was of the same magnitude at 0.052 x 10^{-4}/nt to 0.248 x 10^{-4}/nt (Table II-6).

DISCUSSION

Populations resulting from a homogenous founding haplotype vary stochastically

The unique haplotype compositions observed after serial passages of TriMV populations were diverse among lineages and between passages, with the exception of haplotype t in the CP (Table II-1). These findings are consistent with results found in earlier passage experiments using WSMV (Hall *et al*., 2001; French and Stenger, 2005), where sequence changes were traced from a common source in several lineages. A deterministic outcome would be expected to have caused a similar haplotype distribution within lineages A, B, and C, given that each was subjected to identical selection forces. However, this was not the case, as haplotypes detected within the P1 and CP cistrons were random, with little correlation between lineages or number of passages from the founding population. These results are consistent with stochastic changes due to genetic drift, which occurs in population structures after repetitive and severe bottlenecks.

The prevalence of haplotype A frequency observed in the P1 cistron across passages shows a potential negative selection event taking place within lineages B and C. The population became less heterogeneous with the elimination of the variant haplotypes and selection for the founding haplotype A (Figure II-3 and Figure II-5). This shift towards the founding haplotype A could be driven by the function of TriMV P1, which is responsible for TriMV host RNA silencing suppression (Tatineni *et al.,* 2012). Mutations within this region might be detrimental to the survival of the overall virus population within the plant and thus selection would operate to conserve genomes with non-variant P1 cistrons. Within the CP cistron, divergence from haplotype A was observed over the

three passages for lineages B and C (Figure II-4 and Figure II-5). The haplotype frequencies revealed within the CP cistron might be the result of bottlenecking and genetic drift, since all clones sampled within lineages B and C, by passage 18 and 24 were unique haplotypes. Haplotype A was not observed in any clones sampled from lineage B or C after passage 9.

Selection explains two shared substitutions during passage

The near-universal nucleotide reversion, between guanine and adenine represented a synonymous and a non-synonymous substitution within the CP cistron found to be conserved over passages within the lineages from which they were detected. Non-synonymous mutation of $A_{9423} \rightarrow G$ within the N-terminus of the CP cistron caused an amino acid substitution of lysine for arginine. This mutation, conserved in lineage A, was observed within every passage sampled (Table II-4). These results are most consistent with selection of a neutral mutation that did not reduce fitness. A selective advantage of arginine over lysine at this codon position could reflect equal fitness with the founding inoculum haplotype A. For this particular substitution to be discovered in multiple clones at each passage sampled, its frequency within the TriMV population as a whole must have been large.

A synonymous mutation of G to A at position 9,478 within the CP cistron was detected in both lineages B and C at passages 18 and 24. All 20 clones tested from lineage B and C in passage 18 shared nucleotide A_{9478}. The starting inoculum for each lineage was an infectious clone, therefore the G to A substitution must have occurred *de*

novo in lineage, B and C. In order for each clone sampled to contain this precise substitution, there must be some selective advantage of A over G.

Singletons and non-singletons detected within the TriMV population

Two classes of variation were detected in the P1 and CP cistron data sets: singletons and non-singletons. These two categories have been used to classify heterogeneity within other plant viruses, such as WSMV (Hall *et al.*, 2001; French and Stenger, 2005). Negative strands are not encapsulated, and thus are not removed from the pool of replicating templates. Hence, new mutations created during negative strand production are far more likely to be amplified than a new mutation generated during positive strands synthesis. Mutants must be replicated and able to move within the host to be subjected to selection and drift. Only those mutants that survive lead to a distribution of shared polymorphisms in a sample, these are known as non-singletons. Singletons therefore represent new mutations that have occurred in the positive strand copying from the negative strand template, thus they have not undergone selection.

The observation that the frequency of singletons remained fairly constant over time, is consistent with previous studies of population diversity observed for *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), WSMV and *Zucchini yellow mosaic virus* (ZYMV) (Schneider and Roossinck, 2001; Hall *et al.*, 2001; French and Stenger 2005; Simmons *et al.*, 2011). One probable interpretation of these results is that the majority of singletons are packaged immediately upon generation and therefore do not precipitate in the replication pool. It is not unexpected for the frequency of singletons to remain constant across passages. Due to the high error rate and lack of viral

polymerase proofreading mechanisms, singleton variation is continuously formed and removed from the replicating population. However, it must be noted that, since singletons have not undergone any form of selection, the singleton population could include lethal or deleterious mutations. Substitutions causing stop codons or whole nucleotide deletions within the open reading frame of the P1 and CP cistrons were detected within our data set (Table II-3 and Table II-4).

Mutation frequency per nucleotide of TriMV compared to other plant viruses

The mutation frequency identified within the P1 and CP cistrons of TriMV is low compared to other potyviruses. The mutation frequency within the CP of WSMV after nine serial passages within wheat was 5.8×10^{-4}/nt (Hall *et al.,* 2001; French and Stenger, 2005). For *Zucchini yellow mosaic virus* (ZYMV) and *Turnip mosaic virus* (TuMV) CP cistrons the mutation frequencies were established to be 8.7×10^{-4}/nt and 5.63×10^{-4}/nt/year, respectively (Simmons *et al.,* 2011; Nguyen *et al.,* 2013). Mutation frequencies have also been identified in other non-potyviruses; (+)-sense, single stranded RNA viruses such as *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) 7.0×10^{-4}/nt and 9.0×10^{-4}/nt, respectively (Schneider and Roossinck, 2001). The mutation frequencies discovered in the previous research were found by serially passaging each virus and screening clones, except for TuMV where a Bayesian phylogenetic approach was used to estimate the nucleotide substitution rate. Therefore, a direct comparison of mutation frequency per nucleotide between viruses can be achieved since the methods used were similar.

The mutation frequency discovered for the CP cistron of TriMV at passage 9, of 2.231×10^{-4}/nt, is half that of the mutation frequency found in other viruses (Table II-6). Previous research has shown that the CP cistron is highly conserved in potyviruses. These results suggest the CP cistron is perhaps more conserved within TriMV. The reasoning behind this finding could be a number of evolutionary factors. One possibility is that TriMV is most commonly found co-infected with WSMV, within naturally infected fields, and this interaction could have an impact on its mutation frequency (Byamukama *et al.*, 2013). There is some speculation that TriMV originated within wildgrasses closely related to wheat, making the transition to wheat an easy evolutionary jump. Research has discovered that mutation frequencies vary within TMV and CMV when passed through different hosts (Schneider and Roossinck, 2001).

The mutation frequency per nucleotide for the P1 cistron was not previously determined in WSMV. It is worth noting that the mutation frequency discovered in P1, (0.580×10^{-4}/nt) at passage 9, was lower when compared to the mutation frequency of CP. Previous reports identified P1 to be the least conserved domain in monopartite potyviruses (Valli *et al.*, 2007; Seifers *et al.*, 2013). One possible explanation for our results is that the P1 of TriMV functions in suppression of host RNA silencing, whereas in other potyviruses HC-Pro suppresses host RNA silencing (Kasschau and Carrington, 1998; Tatineni *et al.*, 2012). The low mutation frequency might be the result of the fact the P1 region cannot tolerate lethal or deleterious mutations that hinder the protein's vital function.

Changes upon passages are consistent with genetic drift and negative selection

Changes within the P1 and CP cistrons of TriMV were measured during serial passages at a high MOI and under controlled environmental conditions. It is clear from these results that TriMV populations in a controlled environment are dynamic, with genotypes arising, gaining predominance, losing dominance to other sequences, or serving as the basis for accumulation of further alterations. TriMV population variation was dominated by singletons within the P1 and CP cistrons. Thus, stochastic processes, such as genetic drift are impacting the population. Increasingly, research has shown that stochastic processes are important in plant viral evolution (Choi *et al.,* 2001; Hall *et al.,* 2001; Sacristán *et al.,* 2003; Li and Roossinck, 2004; French and Stenger, 2005; Elena *et al.,* 2008).

It is generally thought that high MOI inoculations should have limited bottlenecking severity. However, our data provides clear evidence in the distribution and composition of variation during passages that severe bottlenecks transpired. Complexity among haplotypes with non-singletons was limited to passage 24, lineage A. Three substitutions created haplotype I of P1 (Table II-2) observed in all 20 clones, with only three having an additional unique mutation. This could have been caused by these substitutions arising within a negative strand template early in the infection process, increasing the occurrence of those mutations in subsequent populations. Non-singletons with multiple additional substitutions were not observed within the CP data set. From these results, we can conclude that severe bottlenecking is occurring within TriMV populations. This is consistent with the results observed earlier on WSMV population dynamics (Hall *et al.,* 2001; French and Stenger, 2005; Bartels, 2011).

Selection and drift are not exclusive forces and may perhaps occur concurrently. One systemic leaf was sampled from each lineage at each time point, thus the TriMV population diversity observed was occurring within a single wheat leaf. Negative selection best explains the trend observed with the frequency of haplotype A in the P1 cistron across passages. However, genetic drift is a plausible explanation for the shift away from haplotype A within the CP cistron. The process of genetic drift is fundamentally random, with the outcome within any single lineage not predetermined. From this research it can be concluded that the combined effect of both forces acting simultaneously on different regions of the genome is what ultimately regulates the degree of variance within virus populations.

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TABLES

Table II-1. Haplotypes determined by SSCP analysis and verifed by sequencing.

Passage	Haplotype[a]			No. of haplotype A[b]
	Lineage: A	B	C	
P1				
9	B, C	D, E, F, G, H	(17) I, J, K, L	33
18	M, N	O, P, Q, R	T, U, V, W, X, Y	48
24	(2) Z, AA, BB	CC	DD	54
CP				
9	b, c, d, e	f, g	h, i, j, k, l, m, n	47
18	o, p, q, r, s	(16) t, u, v, w, x	(17) t, (2) y, z	15
24	aa, bb	(5) t, (4) cc, dd, (8) ee, ff, gg	(19) t, hh	18

For each passage/lineage P1 and CP PCR products were cloned into (pCR-Blunt II-TOPO) and 20 clones were then screened by SSCP.

[a] Letters correspond to unique SSCP patterns for P1 (upper case letters) or CP (lower case letters) cistron regions.

Numbers in parentheses indicate the number of genomes with that unique haplotype.

[b] Number of samples matching the founding inoculum.

Table II-2. Nucleotide substitutions consensus among P1 and CP cistrons of TriMV serially passaged. The haplotypes identifed appeared more than once within a lineage and/or passage. Those amino acids identifed in bold are non-synomous mutations.

Passage / Lineage	# of clones	PCR product	Change from haplotype A[a]			
			Haplotype	nt	Codon	Amino acid
9 / C	17	P1	I	726.1	: → T	T inserted (5' UTR)
				727.1	: → C	C inserted (5' UTR)
				850	AA<u>G</u> → AA<u>T</u>	**Lys to Asn**
24 / A	2	P1	Z	1564	CA<u>T</u> → CA<u>C</u>	His to His
18,24 / B,C	57	CP	t	9478	TT<u>G</u> → TT<u>A</u>	Leu to Leu
18 / C	2	CP	y	9478	TT<u>G</u> → TT<u>A</u>	Leu to Leu
				9790	AG<u>C</u> → AG<u>T</u>	Ser to Ser
24 / B	4	CP	cc	9478	TT<u>G</u> → TT<u>A</u>	Leu to Leu
				9550	GA<u>C</u> → GA<u>T</u>	Asp to Asp
24 / B	8	CP	ee	9478	TT<u>G</u> → TT<u>A</u>	Leu to Leu
				9591	G<u>C</u>G → G<u>T</u>G	**Ala to Val**

[a] The inoculum haplotype is the haplotype of the inoculum used to inoculate passage 1 and used as the SSCP control.

Table II-3. Nucleotide substitutions detected within the P1 cistron after passage 9, 18, and 24. Mutations observed in multiple clones were noted in parentheses and as shared substitutions. Those amino acids noted in bold are non-synomous subititutions.

Passage 9			Passage 18			Passage 24		
Lineage	Substitution	Amino acid change	Lineage	Substitution	Amino acid change	Lineage	Substitution	Amino acid change
A	$C_{576} \rightarrow U$	5'-UTR	A	$U_{458} \rightarrow C$	5'-UTR	A	$U_{1048} \rightarrow C$	Val to Val
	$A_{836} \rightarrow G$	Glu to Glu		$U_{1547} \rightarrow G$	**Phe to Val**		$G_{1503} \rightarrow A$	**Arg to Lys**
	$A_{993} \rightarrow U$	**Lys to Met**					$G_{1556} \rightarrow A$	**Val to Ile**
							$U_{1564} \rightarrow C$	His to His (4)
B	$C_{424} \rightarrow U$	5'-UTR	B	$C_{334} \rightarrow U$	5'-UTR	B	$:_{723.1} \rightarrow U$	5'-UTR
	$U_{625} \rightarrow C$	5'-UTR		$A_{454} \rightarrow U$	5'-UTR		$U_{957} \rightarrow C$	**Met to Thr**
	$G_{931} \rightarrow A$	Val to Val		$G_{918} \rightarrow A$	**Ser to Asn**			
	$G_{960} \rightarrow A$	**Gly to Asp**		$C_{995} \rightarrow U$	**Pro to Ser**			
	$A_{1139} \rightarrow G$	**Arg to Gly**		$C_{1063} \rightarrow U$	Tyr to Tyr			
	$C_{1762} \rightarrow U$	Asp to Asp		$A_{1319} \rightarrow G$	**Asn to Asp**			
				$G_{1364} \rightarrow A$	**Val to Ile**			
				$C_{1448} \rightarrow A$	**Gln to Lys**			
C	$U_{345} \rightarrow C$	5'-UTR	C	$C_{340} \rightarrow U$	5'-UTR	C	$C_{995} \rightarrow U$	**Pro to Ser**
	$:_{726.1} \rightarrow U$	5'-UTR (20)		$U_{348} \rightarrow G$	5'-UTR		$U_{1842} \rightarrow G$	**Leu to Trp**
	$:_{727.1} \rightarrow C$	5'-UTR (20)		$A_{403} \rightarrow :$	5'-UTR			
	$G_{850} \rightarrow U$	**Lys to Asn (20)**		$C_{655} \rightarrow U$	5'-UTR (2)			
	$C_{953} \rightarrow U$	**Pro to Ser**		$U_{968} \rightarrow C$	**Phe to Leu**			
	$G_{1052} \rightarrow A$	**Ala to Thr**		$C_{1023} \rightarrow U$	**Thr to Ile**			
				$G_{1071} \rightarrow A$	**Arg to His**			
				$U_{1201} \rightarrow C$	Asp to Asp			
				$A_{1421} \rightarrow U$	**Lys to stop***			
				$A_{1537} \rightarrow G$	**Ile to Met**			
				$U_{1744} \rightarrow C$	Val to Val			

***** Mutations likely to create a defective virus.

Table II-4. Nucleotide substitutions detected in the CP cistron after passage 9, 18, and 24. Mutations occuring in multiple clones were denoted in parentheses and as shared substitutions. Those amino acids noted in bold are non-synomous subititutions.

Passage 9			Passage 18			Passage 24		
Lineage	Substitution	Amino acid change	Lineage	Substitution	Amino acid change	Lineage	Substitution	Amino acid change
A	$A_{9423} \rightarrow G$	**Lys to Arg (4)**	A	$G_{9100} \rightarrow A$	Val to Val	A	$A_{9423} \rightarrow G$	**Lys to Arg (2)**
	$G_{9506} \rightarrow A$	**Ala to Thr**		$C_{9106} \rightarrow A$	**Phe to Leu**		$U_{9630} \rightarrow C$	Val to Ala
	$C_{9651} \rightarrow U$	**Ala to Val**		$A_{9423} \rightarrow G$	**Lys to Arg (5)**		$U_{9835} \rightarrow C$	Ala to Ala
	$G_{9817} \rightarrow U$	Pro to Pro		$U_{9927} \rightarrow C$	**Val to Ala**			
	$C_{9917} \rightarrow U$	**Arg to Stop**		$C_{9954} \rightarrow U$	**Pro to Leu**			
B	$G_{9315} \rightarrow A$	**Gly to Asp**	B	$C_{9409} \rightarrow U$	Ile to Ile	B	$A_{9242} \rightarrow C$	**Thr to Pro**
	$U_{9634} \rightarrow C$	Asn to Asn		$G_{9478} \rightarrow A$	Leu to Leu (20)		$G_{9478} \rightarrow A$	Leu to Leu (20)
				$C_{9504} \rightarrow U$	**Ala to Val**		$G_{9489} \rightarrow U$	**Gly to Val**
				$G_{9817} \rightarrow :$	**frameshift***		$C_{9537} \rightarrow U$	**Ala to Val**
				$C_{9818} \rightarrow :$	**frameshift***		$C_{9550} \rightarrow U$	Asp to Asp (4)
				$A_{9822} \rightarrow G$	**Asn to Ser**		$C_{9591} \rightarrow U$	**Ala to Val (8)**
				$G_{9892} \rightarrow A$	Gln to Gln			
C	$U_{9209} \rightarrow A$	**Ser to Thr**	C	$G_{9326} \rightarrow U$	**Asp to Tyr**	C	$G_{9478} \rightarrow A$	Leu to Leu (20)
	$A_{9222} \rightarrow U$	**Lys to Met**		$G_{9478} \rightarrow A$	Leu to Leu (20)		$U_{9485} \rightarrow C$	**Phe to Leu**
	$U_{9328} \rightarrow A$	**Asp to Glu**		$C_{9790} \rightarrow U$	Ser to Ser			
	$C_{9429} \rightarrow U$	**Ala to Val**		$C_{10005} \rightarrow U$	**Thr to Met**			
	$C_{9448} \rightarrow U$	Asp to Asp						
	$U_{9539} \rightarrow C$	**Phe to Leu**						
	$G_{9848} \rightarrow A$	**Val to Thr**						
	$U_{9849} \rightarrow C$	**Val to Thr**						
	$A_{9873} \rightarrow G$	**Asp to Gly**						
	$U_{10009} \rightarrow A$	**Asn to Lys**						

* Mutations likely to create a defective virus.

Table II-5. Detectable errors induced by *Pfu* Ultra II polymerase during PCR of the P1 or CP cistron was established.

PCR product of plasmid	No. of clones SSCP screened	No. of polymorphic sites/no. of nt	Mutation frequency/nt
P1	14	1/21,406	0.46716×10^{-4} /nt
CP	14	0/14,070	-

Table II-6. Nucleotide polymorphisms discovered within the P1 and CP cistron after 9, 18, and 24 passages.

PCR product	Passage	No. of polymorphic sites/no. of nt	Mutation frequency/nt	Corrected total*	Mutation Frequency/nt /passage
P1	P9	15/91,740	1.635×10^{-4}	0.580×10^{-4} [a]	0.064×10^{-4}
	P18	21/91,740	2.289×10^{-4}	1.234×10^{-4} [a]	0.069×10^{-4}
	P24	8/91,740	0.087×10^{-4}	-	-
CP	P9	17/60,300	2.819×10^{-4}	2.231×10^{-4} [a]	0.248×10^{-4}
	P18	16/60,300	2.653×10^{-4}	2.065×10^{-4} [a]	0.115×10^{-4}
	P24	11/60,300	1.824×10^{-4}	1.236×10^{-4} [a]	0.052×10^{-4}

* RT-PCR error for P1 was 1.055×10^{-4}/nt, with the RT error of 0.588×10^{-4}/nt and the *Pfu* II Ultra DNA polymerase error of 0.467×10^{-4}/nt. The RT-PCR error for CP was only the RT error of 0.588×10^{-4}/nt, since no PCR error was discovered for CP.

[a] No significant difference using an Two-Way ANOVA ($P < 0.05$)

FIGURES

Figure II-1

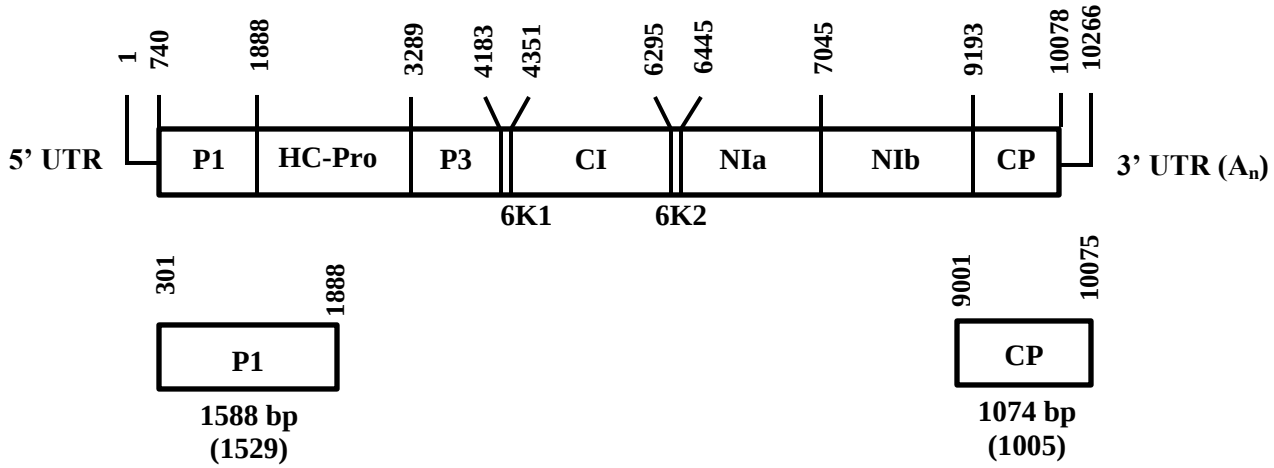

Figure II-1. Physical map of the TriMV genome illustrating location of RT-PCR products amplified for examination of genotypic variation. Cistron arrangement within the polyprotein open reading frame bracketed by the 5' and 3' untranslated regions is displayed at the top. The RT-PCR product nucleotide lengths and nucleotide sequence lengths analyzed for nucleotide polymorphism are presented below.

Figure II-2

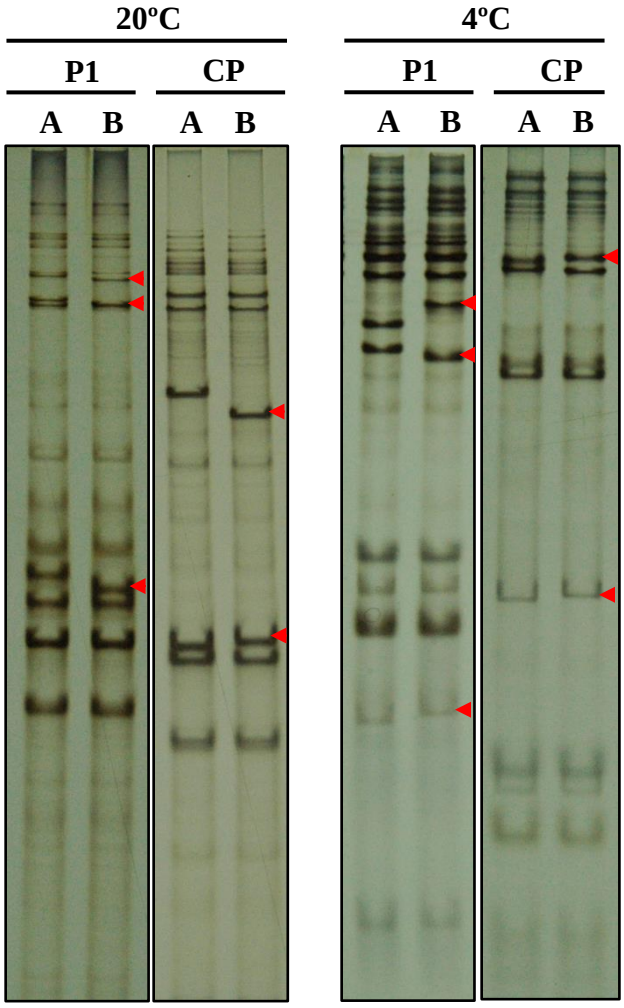

Figure II-2. TriMV coat protein (CP) and protein 1 (P1) cistrons analyzed by SSCP assay. SSCP profiles display haplotype A in the inoculum and the observed haplotype B in passage 9. PCR products denatured and digested with restriction enzymes were electrophoresed on 10% polyacrylamide gels at 20ºC and 4ºC under nondenaturing conditions. SSCP patterns unlike the haplotype A inoculum pattern at one or both temperatures examined, corresponded to a new haplotype.

Figure II-3

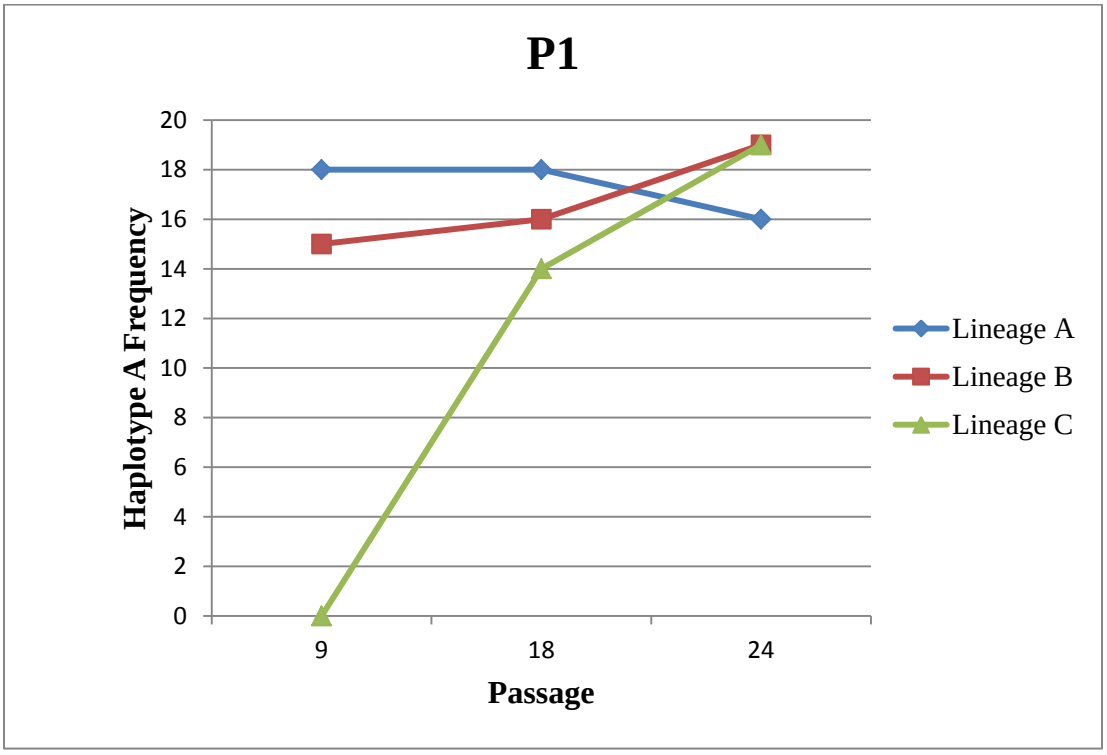

Figure II-3. The graph displays the frequency of haplotype A within lineages A, B, and C for the P1 cistron at passage 9, 18, and 24. Lineage B and C increased the number of clones found to be haplotype A as passages increased. The frequency of haplotype A in lineage A at passage 9 and 18 remained constant at 18, at passage 24 the number of clones representing haplotype A dropped slightly to 16.

Figure II-4

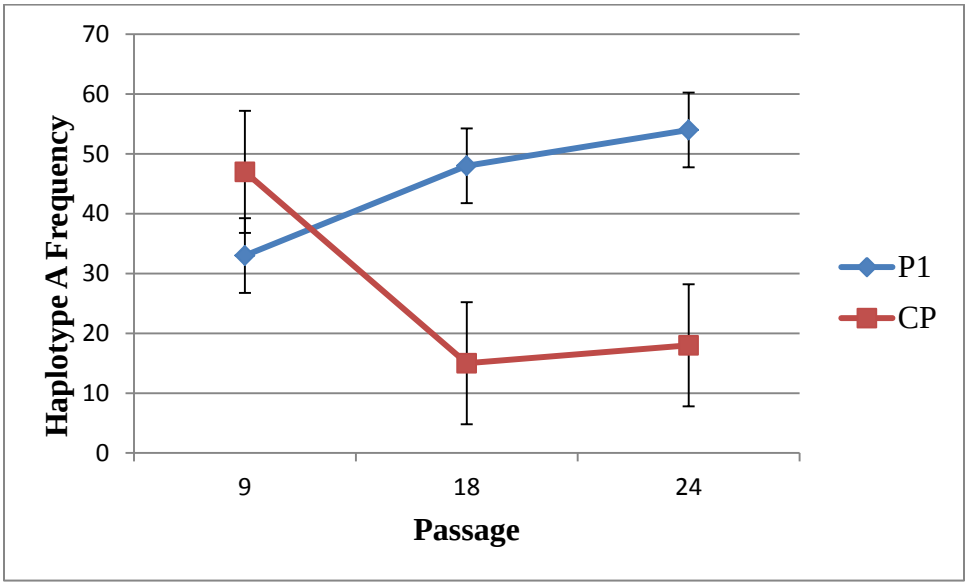

Figure II-4. By treating lineages A, B, and C as replications in the number of clones found to be haplotype A can be seen across passages 9, 18, and 24. The P1 cistron displays an increase in haplotype A across the three passages, whereas the CP cistron shows a decrease in haplotype A as the number of passages increased.

Figure II-5

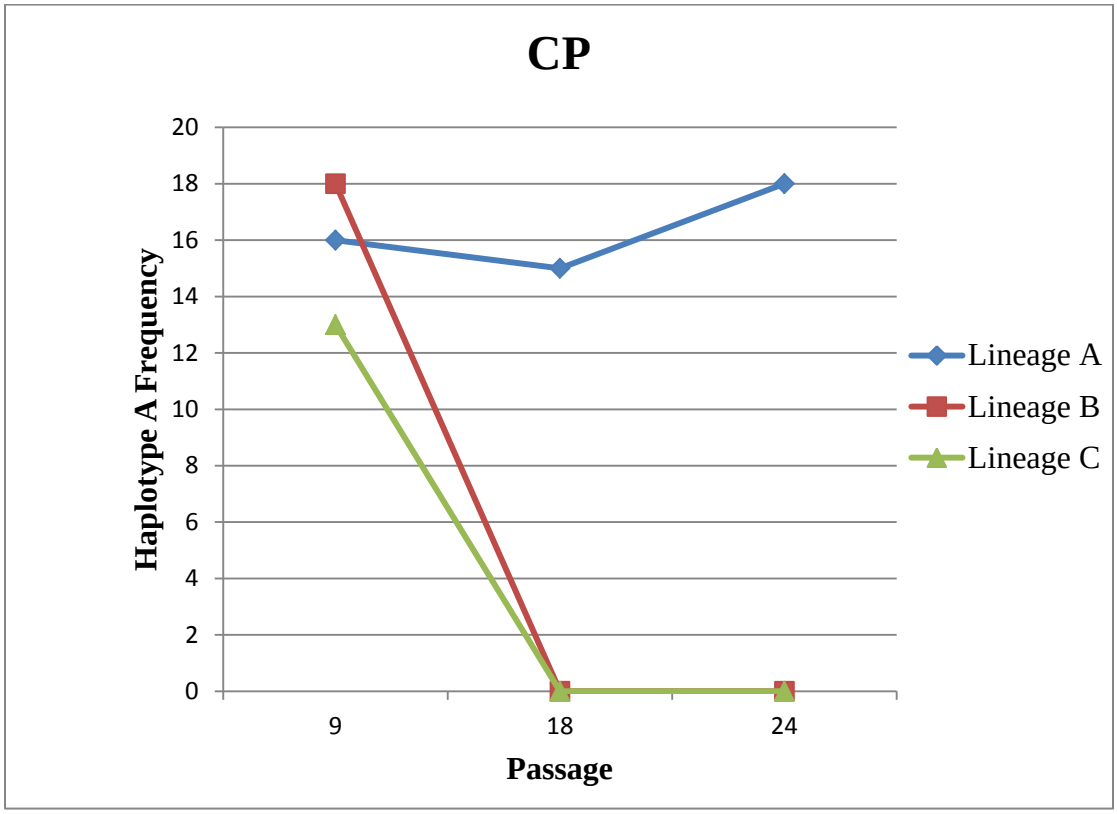

Figure II-5. The graph displays the frequency of haplotype A for the CP cistron over passages 9, 18, and 24. The decrease in haplotype A can be seen across passage 9, 18, and 24 for lineage B and C. The frequency of haplotype A within lineage A remained steady from passage 9 to 18, a slight increase was observed in passage 24.

CHAPTER 3

Variation Within the Coat Protein of *Triticum Mosaic Virus* is Not Determined by Variant Host Species

ABSTRACT

Previous research has established that the mutation frequencies of RNA plant viruses can vary with changing environments (e.g., different hosts). To determine whether these observations extend to the newly discovered virus *Triticum mosaic virus* (TriMV), experiments were conducted evaluating passage through alternative hosts. Two isolates of TriMV were inoculated onto four hosts, wheat (*Triticum aestivum cv.* Tomahawk), barley (*Hordeum vulgare cv.* Metcalfe), rye (*Secale cereale cv.* Petkus), and triticale ([X *Triticosecale* Wittmack] line NE 422T), to investigate the effect of environmental changes on the mutation frequencies of the coat protein (CP) cistron of TriMV. Viral RNA was extracted from each host and cloned; each clone represented a unique viral RNA, allowing a snapshot of the genetic diversity generated within a given viral population. By comparing the sequence of the viral clones by single-strand conformational polymorphism (SSCP) to the inoculum sequence the mutation frequency of CP within each host could be established. The mutation frequencies observed in the CP of TriMV_4685 isolate over the various hosts ranged from 0.656×10^{-4}/nt to 1.153×10^{-4}/nt. For TriMV_5262 isolate, the mutation frequencies of CP detected from the various host species were 0.407×10^{-4}/nt to 0.656×10^{-4}/nt. No significant differences between isolates in their mutation frequencies were found within the host species; hence, in nature, their base rate of diversity should be similar. A co-inoculation study found that the two TriMV isolates were distributed relatively evenly within clones obtained from TriMV populations extracted from the various hosts. This suggests the isolates examined are equally adapted to the four hosts. Results suggest natural TriMV populations maintain genetic stability within the CP cistron.

INTRODUCTION

Population genetic diversity is a key aspect in allowing species to evolve in diverse environments with ever-changing selection pressures. Viruses have adapted to parasitize most identified organisms due to their extreme evolution capabilities. RNA viruses exhibit both error-prone replication and short replication time which can result in diverse populations (Holland *et al.,* 1982; Novella *et al.,* 1995; McNeil *et al.,* 1996; Hall *et al*., 2001b; Harrison, 2002; French and Stanger, 2003; García-Arenal *et al.,* 2003; Elena *et al.,* 2008; Gao and Feldman, 2009; Tromas and Elena, 2010). Sequence diversity in plant RNA viruses has been established between isolates, although within individual isolates, low levels of variation have been found (Kurath and Palukaitis, 1990; Albiach-Martí, *et al*., 2000; Kong *et al.,* 2000; French and Stenger, 2003; García-Arenal *et al.,* 2003). Therefore, evolutionary factors, such as genetic bottlenecking and negative selection, must play a role in favoring genetic stability for many plant viruses (Hall *et al.,* 2001b; Schneider and Roossinck 2001; García-Arenal *et al.,* 2003; Li and Roossinck, 2004; French and Stenger, 2005; Acosta-Leal *et al.,* 2011).

Previous research has established that the mutation frequencies of RNA plant viruses can vary with different hosts. *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) displayed significantly different mutation frequencies within alternate host species (Schneider and Roossinck, 2001). By assessing the mutation frequency of a virus within different hosts, the potential level of diversity can be determined. For both TMV and CMV, low genetic diversity was established in tomato, while high genetic diversity was displayed within the virus populations isolated from

pepper plants (Schneider and Roossinck, 2001). More research is needed to fully understand the various evolutionary aspects affecting plant RNA viruses within their host species.

The variation of *Triticum mosaic virus* (TriMV) populations within different host species was addressed in this research. TriMV is a type member of the genus *Poacevirus* within the family *Potyviridae* (Seifers *et al.,* 2008; Fellers *et al.,* 2009; Tatineni *et al.,* 2009; Adams *et al.,* 2011). TriMV is a (+)-sense, single-stranded RNA virus, consisting of 10,266 nucleotides, not including the poly A tail at the 3' end (Fellers *et al.*, 2009; Tatineni *et al*., 2009). The genome is translated as a single polyprotein that is cleaved by three viral proteinases (P1, HC-Pro and NIa) into 10 mature proteins (Fellers *et al.,* 2009; Tatineni *et al*., 2009). TriMV is vectored by the wheat curl mite (*Aceria tosichella*), another potential source of bottlenecking for viral populations (Seifers *et al.,* 2009). TriMV isolates from wheat field samples within the Great Plains region were established to have little variation (Fuentes-Bueno *et al.,* 2011; Seifers *et al.,* 2013).

Two TriMV isolates used in this research have been identified in field samples across the Great Plains region with no geographical pattern to their distribution (French, unpublished). The isolates have a single nucleotide difference in their coat protein (CP) at nucleotide position 9,242, causing an amino acid change. The TriMV isolates were inoculated singly and co-inoculated on four different host species to investigate the effect of environmental changes on the mutation frequency of TriMV CP cistron. Regions of the CP cistron are known to be highly conserved within the *Potyviridae* family. The CP cistron contains major virus-specific potyvirus epitopes (Shukla *et al.,* 1988). Within

isolates of WSMV, the N- and C-terminal regions of CP are responsible for host specificity in long distance movement (Tatineni *et al.,* 2011; Tatineni *et al.,* 2014).

This experiment utilized the single-strand conformation polymorphism assay (SSCP) to identify the variation within the CP cistron. SSCP is a tool utilized to expose variations within virus populations (Orita *et al.,* 1989; Rubio *et al,* 1996; Welsh *et al.,* 1997; Pawlotsky *et al.,* 1998; Hall *et al.,* 2001b; Kong *et al.,* 2003; Gasser *et al.,* 2006; Goszczynski, 2007). SSCP enabled polymorphisms to be detected in the coat protein (CP) of TriMV across multiple samples. This research determined the variation of TriMV populations between host species and preference of the two TriMV isolates among wheat, rye, barley, and triticale.

MATERIAL AND METHODS

TriMV field isolates

TriMV isolates were identified from field samples by Dr. Dallas Seifers from Kansas State University. The CP cistron was amplified by RT-PCR and sequenced previously (French, unpublished). Sequences revealed a non-synonymous substitution at nucleotide position 9,242 within the CP cistron. At nucleotide position 9,242, isolate 4685 has ACA coding for threonine identical to the previously sequenced CP region of the Nebraska strain of TriMV (GenBank, accession no. FJ669487) (Tatineni *et al.,* 2009). TriMV isolate 5262 has GCA coding for amino acid alanine matching the CP region of the Kansas strain of TriMV (GenBank, accession no. FJ263671) (Fellers *et al.,* 2009).

Inoculum production

Triticum aestivum cv. Tomahawk (wheat) seeds were planted in six-inch diameter clay pots, approximately 16 to 18 seeds per pot, in a pre-mixed sterilized potting medium. Plants were grown in a greenhouse at 20˚C to 26˚C and watered daily to minimize differential selective pressures due to environmental conditions. Each pot was thinned to 15 wheat plants at seven to nine days old before inoculation. A 1:10 dilution of plant sap inoculum was prepared by grinding one gram of leaf tissue of TriMV_4685 or TriMV_5262 isolates in 10 ml of sterile water. TriMV_4685 and TriMV_5262 inoculum was mechanically inoculated onto four pots of wheat plants. Whole inoculated wheat plants (excluding the roots) were collected at 14 days post inoculation (dpi), placing six plants per bag, and stored at -80°C.

Host plants

Four host species were selected: wheat cv. Tomahawk, barley (*Hordeum vulgare cv.* Metcalfe), rye (*Secale cereale cv.* Petkus), and triticale ([X *Triticosecale* Wittmack] line NE 422T). These particular cultivars were utilized due to their susceptibility to TriMV (Tatineni *et al.,* 2010). Fifteen seeds were planted in six-inch diameter clay pots containing pre-mixed potting medium. Six pots of each host plant were planted, two pots for each TriMV isolate being tested and two pots for co-infection. Plants were grown in the greenhouse at 20˚C to 26˚C and watered daily. Pots were thinned to 20 plants per host before inoculation.

Inoculation of different host species

For single inoculations, one TriMV isolate (4685 or 5262), was used to infect two pots of wheat, barley, rye, and triticale, for a total of eight inoculated pots per isolate. The inoculum was prepared by grinding six leaf samples (~ 2.0 grams of tissue kept at -80˚C) in 20 ml of sterile water (1:10 dilution). For double inoculations containing both TriMV isolates 4685 and 5262, 10 ml of each single inoculum were mixed to obtain a 1:20 dilution. The co-infection inoculum was used to infect two pots of wheat, barley, rye, and triticale. Five hundred microliters of each isolate's inoculum and co-infection inoculum was collected to be used as positive controls (founding inoculum) for SSCP analysis. Plants were mechanically inoculated at seven to nine days old, at the emergence of the two-leaf stage.

Total RNA extraction

For each TriMV isolate used for single infection and the co-infections, two upper systemic leaves from five plants were collected from each host for analysis at 14 dpi. Viral RNA was isolated by phenol:chloroform extraction as follows: samples were placed in a grinding bag with 1 ml of sterile water and hand ground until the leaves became slightly translucent. For each sample, 250 µl of leaf extract was transferred to a fresh 1.5 ml tube containing 250 µl of 2x glycine buffer (pH 9.0) (0.2 M Glycine, 20 mM EDTA, 0.2 M NaCl), 25 µl of 20% SDS and 500 µl of phenol:chloroform:isoamyl alcohol. The samples were vortexed and centrifuged at 13,200 rpm for five minutes. Roughly, 300 µl of supernatant was transferred to a fresh 1.5 ml tube on ice, where 30 µl of 3M sodium acetate (pH 4.8) and 900 µl of 100 % ethanol were added, and tubes inverted. Samples were centrifuged at 13,200 rpm for five minutes at 4ºC. The pellet was washed with 500 µl of 70% ethanol, vacuum dried and resuspended in 25 µl of sterile water. The extracted total RNA was stored at -80˚C.

Reverse transcription (RT) and polymerase chain reaction (PCR)

The CP cistron was amplified by RT-PCR using CP specific primers. Primers annealing to the CP region of TriMV were comprised of a forward primer, Tr-89, annealing to nucleotides 9,001 to 9,019, 5’-CAAGATTAACGCGGCATGG-3’and a reverse primer, Tr-100, annealing to nucleotides 10,048 to 10,075, 5’-AACCTCGAGCTAACGGGTACCAAACATGGCCCCGCCGACA-3’.

Viral RNA was transcribed at 42˚C for 1 hour, followed by 99˚C for 5 min and held at 10˚C. Each reverse transcription reaction consisted of 5.9 µl of sterile water, 2 µl

of 5x buffer (RT AMV), 0.5 µl of 50 ng/µl virus CP specific reverse primer, 0.4 µl of 10 mM dNTPs, 0.2 µl of RT AMV (Roche, Indianapolis, IN) (23 U/µl) and 1 µl of total RNA. PCR targeting the CP region was performed on all samples using high fidelity *Pfu* II Ultra DNA polymerase (Agilent Technologies). One µl cDNA was used for PCR per 25 µl reaction volume with CP specific primers. The PCR program consisted of 95˚C for 2 min followed by 10 cycles at 95˚C for 30 s, 48˚C for 30 s and 72˚C for 2 min. The annealing temperature was raised to 56˚C for the remaining 30 cycles. The PCR program consisted of 40 cycles on a T1 thermocycler (Biometra). The RT-PCR products were visualized by 1.0% agarose gels in TBE (Tris-borate-EDTA) buffer.

Cloning of PCR products

A Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Life Technologies) was utilized to clone CP PCR products obtained from either TriMV single or co-infected plants from each host into One Shot chemically competent *Escherichia coli*. The manufacturer's protocols were followed as provided. Two clones were selected from each transformation, and the following miniprep protocol was used to isolate the plasmids. A single colony was used to inoculate 2 ml of LB media (containing kanamycin) in a 10 ml plastic tube; samples were incubated overnight at 37°C and ~220 rpm. The culture was vortexed and poured into a 1.5 ml microfuge tube centrifuged at 13,200 rpm for two minutes. The media was decanted, and 210 µl STET solution (10 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 8% glucose, 0.5% Triton X-100) was added, and placed on a mixer for five minutes to dissolve the pellet. To each sample 15 µl TE buffer containing 15 mg/ml lysozyme was added and vortexed, the tubes were

boiled for 50 seconds, then centrifuged at 13,200 rpm for 15 minutes. Pellets were removed and 150 µl of 7M ammonium acetate (pH 7.2) and 800 µl 100% ethanol was added, then mixed by inversion and incubated at -20°C for ten minutes. Samples were centrifuged at 13,200 rpm for 15 minutes, and pellets were washed with 500 µl 70% ethanol. Samples were then centrifuged at 13,200 rpm for five minutes, pellets were vacuum dried and resuspended in 60 µl sterile water. Plasmids containing the cloned fragment were digested with restriction enzyme *Eco*RI (BioLabs, New England) at 37˚C to release the PCR fragment. Digested plasmids were diluted with sterile water 1:500, and re-amplified by PCR using the previously described reaction mixture and program with the CP specific primers.

Single-strand conformation polymorphism

SSCP analysis relies on PCR which results in the predominant sequence in the sample to be measured. Electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is highly dependent on its size and structure, thus the basis of SSCP (Orita *et al.,* 1989; Gasser *et al.,* 2006). Previous research has established the accuracy of SSCP detection in the CP region of *Wheat streak mosaic virus* (WSMV), also a member of the *Potyviridae* family, to be 95% (Hall *et al.,* 2001b).

Single strand conformation polymorphism was performed on two clones from each single or co-infected host sample. The final PCR product was digested with the appropriate restriction enzyme to form ~200 to 500 bp fragments (Orita *et al*., 1989). For CP, *Dde* I was used, cutting CP into four fragments. Digested samples were prepared for SSCP as described by Hall *et al.,* (2001b). Because the accuracy of SSCP to detect single

nucleotide polymorphisms improves to ~98% when gels are electrophoresed at two different temperatures (Welsh *et al.,* 1997), gels were run at 20ºC and 4ºC at 60 Volts for 16 ½ hours and 45 Volts for 15 ½ hours, respectively, in 1X TBE buffer. Silver staining of the SSCP gels was done using GE Healthcare DNA Silver Staining Kit PlusOne (Waukesha, WI) the protocol was followed as written (Pawlotsky *et al.,* 1998). The undigested plasmids of samples testing positive for conformational polymorphisms were prepared for sequencing.

Plasmids prepared for sequencing

The miniprep DNAs (30 µl) were resuspended in 170 µl of Tris-EDTA buffer (pH 8) and treated with 2 µl RNase A (1ng/µl) for 20 minutes at 37ºC, and then cleaned by phenol:chloroform extraction as follows. Sterile ultra-purified water was added to each sample to bring the volume to 300 µl, 300 µl of phenol:chloroform was added and vortexed thoroughly, and then centrifuged at 13,200 rpm for four minutes. The aqueous phase was collected and 400 µl of chloroform:isoamyl alcohol (24:1) was added to each sample. Samples were mixed and centrifuged at 13,200 rpm for four minutes. The aqueous phase was collected, to which 1/10 the volume (~25 µl) of 3M sodium acetate (pH 4.8) and 2.5 volume (~625 ml) of 100% ethanol was added. Samples were incubated at -70°C for 15 minutes, and then centrifuged at 13,200 rpm for 15 minutes. Supernatant was decanted and the pellet was washed with 500 µl of 70% ethanol, mixed and centrifuged at 13,200 rpm for ten minutes. Supernatant was decanted and the pellet was vacuum dried, then resuspended in 25 µl water. Samples were sequenced at the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida.

Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI) was used to analyze the sequence results. This experiment was replicated twice following the methods described above.

Mutation frequencies

The mutation frequency was calculated by taking the number of polymorphisms discovered by single-strand conformational polymorphism (SSCP) and confirmed by sequencing, dividing by the total number of nucleotides screened. The internal error rate caused by the RT-PCR system used in this research was calculated in chapter 2. The mutation frequencies calculated were corrected by subtracting the RT-PCR error rate of 0.588×10^{-4}/nt, to obtain the TriMV polymerase mutation frequencies per host species.

Statistical Analysis

Statistical analysis was completed using SAS version 9.2 (SAS Institute Inc.). Two-way Analysis of Variance (ANVOA) and Chi-Square tests were used to address differences within and between passages, lineages, and/or proteins.

RESULTS

Mutation frequencies observed in TriMV isolates 4685 and 5262

Each clone represented a unique viral RNA, allowing a snapshot of the genetic diversity generated within a given viral population to be assessed by comparing the sequence of the viral clones to the known inoculum sequence. The mutation frequency of TriMV_4685 from two clones acquired from a single plant of barley, rye, triticale, or wheat is displayed in Table III-1. TriMV_4685 population diversity fluctuated among plants of each host species. For barley, mutations were found in three plants out of the ten sampled. Seven substitutions were identified in barley inoculated with TriMV_4685, producing a mutation frequency of 1.153×10^{-4}/nt (Table III-1). For rye, a total of five mutations were detected within three plants, giving a mutation frequency of 0.656×10^{-4}/nt. For triticale, five mutations were discovered in three plants of the sample set, providing an overall mutation frequency of TriMV_4685 of 0.656×10^{-4}/nt. Only three mutations were detected within the CP of TriMV_4685 populations extracted from three wheat plants, providing a frequency of 0.162×10^{-4}/nt. Mutation frequencies of TriMV_4685 were compared between hosts using a Two-way Analysis of Variance (ANVOA) test in SAS version 9.2 (SAS Institute Inc.) with no significant difference ($P < 0.05$).

The TriMV_5262 mutation frequency obtained from a single plant of barley, rye, triticale, or wheat is presented in Table III-2. The population diversity of TriMV_5262 varied among single plants of each species. However, mutations were not observed in TriMV populations obtained from every plant sampled from the various hosts. Five

mutations were found within the CP region of TriMV_5262 extracted from five barley plants, producing a mutation frequency of 0.656×10^{-4}/nt (Table III-1). A mutation frequency of 0.407×10^{-4}/nt was calculated from the four mutations detected within four rye plants. Four mutations were identified in virus isolated from two triticale plants, giving a mutation frequency of 0.407×10^{-4}/nt. Within wheat, five mutations were identified within the CP region of TriMV_5262 from four plants, providing a frequency of 0.656×10^{-4}/nt. A Two-Way ANOVA test ($P < 0.05$) was used to compare the mutation frequencies of TriMV_5262 between hosts, and no significant difference was found. A comparison between TriMV_4685 and TriMV_5262 within each host was completed using a Two-way-ANOVA test. The mutation frequencies obtained from TriMV_4685 and TriMV_5262 isolates from barley, rye, triticale, or wheat were compared statistically, and no significant difference was found.

Mutations within the CP of TriMV_4685 isolate

The mutations observed within the CP of TriMV_4685 obtained from barley, rye, triticale, and wheat are presented in Table III-3. More mutations were detected in TriMV_4685 populations acquired from barley compared to the other hosts. Eleven mutations were discovered in TriMV populations acquired from barley, six being non-synonymous and four being synonymous mutations (Table III-3). The remaining mutation was a deletion that arose in the C-terminus of the CP, and likely would result in a defective viral genome. A synonymous mutation of $C_{9151} \rightarrow U$ within the aspartic acid codon was found in four clones obtained from barley, three from rye, and one from triticale (Table III-3). At nucleotide position 9,151, TriMV_4685 codes for a C where

TriMV_5262 codes for a U. A non-synonymous mutation of $G_{9478} \rightarrow A$, causing an amino acid substitution of glutamic acid for lysine, was detected in four clones from barley, and three from rye (Table III-3). These two mutations, detected within TriMV_4685 populations obtained from different hosts, were found together within the same clone seven out of eight times. Four clones acquired from barley had both of the above mutations, while three of the clones from rye also had these two mutations found together. Seven mutations were detected in TriMV CP samples obtained from rye, four non-synonymous and three synonymous. A total of five mutations were discovered in samples extracted from triticale, with four being non-synonymous and one being synonymous. Wheat had the lowest number of mutations at three positions, two were non-synonymous and one was synonymous. Of the 26 mutations found in TriMV_4685 in all four hosts, transitions were the predominant type of substitutions, meaning a substitution of a purine for a purine or a pyrimidine for a pyrimidine. The exceptions were $G_{10037} \rightarrow U$ from TriMV populations from rye and $C_{9806} \rightarrow A$ from triticale, which were transversions (purine ↔ pyrimidine). Non-synonymous substitutions were predominant in each host over synonymous for TriMV_4685. Overall the mutations observed in the CP of TriMV_4685 did not seem to be biased to any particular regions.

Mutations within the CP of TriMV_5262 isolate

Mutations within TriMV_5262 CP obtained from barley, rye, triticale, and wheat are presented in Table III-4. Five substitutions were identified in TriMV_5262 in barley plant samples with three found to be non-synonymous with two being synonymous. All substitutions occurring in TriMV_5262 from barley were unique transitions. Within the

TriMV population extracted from rye, four mutations were detected, three were non-synonymous and one was synonymous, all of which were only observed once. Three transition substitutions and one transversion substitution were observed in rye. A total of four mutations were discovered in triticale samples, with two being non-synonymous and one being synonymous. The remaining mutation observed was a deletion that occurred in the middle of the CP region, likely resulting in a frameshift producing a defective virus. Two transversion and one transition substitution were identified in triticale. Within wheat five mutations occurred, two being non-synonymous and two being synonymous. A deletion mutant was found towards the C-terminus of the CP, possibly resulting in a defective viral genome. For wheat there were two transversion and two transition substitutions. Non-synonymous substitutions predominated in each host over synonymous substitutions for TriMV_5262, except for wheat where the ratio was even. The mutations detected within the CP of TriMV_5262 did not show a bias to any particular region. All mutations discovered were unique to the host, plant, and clone from which they were acquired.

Occurrence of TriMV_4685 and TriMV_5262 within the same host species

TriMV_4685 and TriMV_5262 were co-inoculated onto barley, rye, triticale, and wheat, to assess if one isolate was better adapted over the other in different hosts. For each experimental replication, five host plants were collected, cloning two viral genomes from each TriMV population isolated per plant. SSCP was utilized to determine if the clones matched the CP regions of isolate TriMV_4685 or TriMV_5262. By using the single inoculum of each isolate and the mixed inoculum as positive controls for SSCP

analysis, each clone could be identified as one of the isolates by differences in band patterns, displayed in Figure III-1. For replication 1 within barley, five clones were TriMV_4685 and five were TriMV-5262, with one plant having both isolates detected systemically (Table III-5). A bias was seen in the predominant isolate within TriMV populations from rye, with one clone identified as TriMV_4685 and the remaining nine as TriMV_5262. Both isolates were obtained from a single rye plant. For TriMV populations acquired from triticale, six samples were found to be TriMV_4685, with four samples were identified as TriMV_5262. The isolates were not detected co-infecting the same triticale plant. For wheat, it appeared TriMV_5262 was possibly the predominant isolate, with seven clones, with three clones detected as TriMV_4685. Of the three clones of TriMV_4685 that were identified in wheat, each was found co-infected with TriMV_5262.

A different trend was observed in replication 2, with no TriMV isolate bias found in barley, rye or wheat (Table III-5). Of the clones obtained from barley TriMV populations, five of each TriMV isolate were detected. For rye, five TriMV_4685 clones were identified, with the remaining five being TriMV_5262. TriMV_4685 occurred in seven of the clones sampled from triticale TriMV populations, while TriMV_5262 was identified in three clones. Both isolates were detected equally in TriMV populations extracted from wheat. For replication 2, for each host systemic leaves from one plant were discovered to be co-infected with both isolates.

DISCUSSION

Mutation frequencies

The mutation frequencies of TriMV isolates observed in different hosts revealed genetic stability of the CP sequence from TriMV populations across host species. The overall mutation frequencies obtained from TriMV_4685 and TriMV_5262 isolates were not significantly different among barley, rye, triticale, or wheat. This could possibly mean that the evolutionary factors influencing the viral population must be similar or identical in the host species tested. Low mutation frequencies detected within TriMV populations are in agreement with previous reports of little genetic diversity among TriMV isolates obtained from field samples (Fuentes-Bueno *et al.*, 2011). Little genetic variation was found between hosts species in *Citrus leaf blotch virus* (CLBV), a (+)-sense, single-stranded genomic RNA (Vives *et al.*, 2002). The observed low mutation frequency in TriMV in different host plants would result in little genetic diversity among TriMV isolates, because new mutations are not arising as fast as in a virus such as *Wheat streak mosaic virus* (WSMV), with a mutation frequency of 5.8×10^{-4} in wheat (Hall *et al.*, 2001b; French and Stenger, 2005). Our results are different from previous research of *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) which identified significantly different mutation frequencies within alternate host species (Schneider and Roossinck, 2001).

It is important to note, variation was observed in the mutation frequency between individual plants of the same host. The diversity between individual plants of the same host is not surprising, since it is known genetic bottlenecking events leading to genetic

drift are random evolutionary factors affecting viral populations (Choi *et al.*, 2001; Hall *et al.*, 2001b; Sacristán *et al.*, 2003; Li and Roossinck, 2004; French and Stenger, 2005; Elena *et al.*, 2008). These random events could drive diversity in one plant, while in other plants genetic diversity is reduced or maintained. Selection and drift are not exclusive forces, and may perhaps occur concurrently (García-Arenal *et al.*, 2003; French and Stenger, 2005; Ali *et al.*, 2006). Therefore, it is reasonable that negative selection is working to maintain genetic stability of TriMV populations, in addition to genetic bottlenecking.

Mutations found in the CP cistron

The mutations observed within TriMV_4685 displayed a pattern. The synonymous C to U substitution at nucleotide position 9,151 was detected in barley, rye, and triticale, but not in wheat. At nucleotide position 9,151, TriMV_4685 codes for a C where TriMV_5262 codes for a U. The substitution was unidirectional from isolate TriMV_4685 to TriMV_5262 at this position. It is plausible that nucleotide U has a selective advantage over C at this position, especially since this precise mutation was identified in the majority of the clones observed in barley and rye. One might think the host could be driving the selection; however, since this is a silent mutation, it is most probable that the selection is working at the RNA level.

The near-global nucleotide reversion from guanine to adenine represented a non-synonymous substitution conserved within the CP cistron of TriMV_4685 extracted from barley and rye. This mutation was discovered in almost all of the clones obtained from TriMV populations extracted from barley and rye. Selection within these hosts might be

driving this particular change in amino acid within the CP. This specific non-synonymous mutation was only detected in conjunction with the silent mutation of C to U as discussed above. It is possible that these two mutations might have caused a conformational change in the RNA that was selected for within these hosts.

The mutations detected in TriMV_5262 populations were sporadic and unique for each host and clone. No pattern between or within hosts could be identified. The mutations discovered are feasibly the result of severe bottlenecking, leading to genetic drift, allowing these mutations to occur at a high enough frequency to be identified within the TriMV population. The ratios of non-synonymous to synonymous mutations were relatively the same across host species for both TriMV isolates. Therefore, we cannot disregard the idea of selection also being an evolutionary force on TriMV populations.

Geographical dispersion

One of the purposes of this research was to explain the simultaneous occurrence of two isolates of TriMV in field samples across the Great Plains region with no distinctive geographical pattern to their distribution (French, unpublished). We speculated that perhaps one TriMV isolate was better adapted to a certain host or hosts over another, resulting in both being conserved within the TriMV population as a whole. TriMV_4685 is identical to the Nebraska TriMV isolate, while isolate TriMV_5262 is identical to the Kansas TriMV isolate, within their CP region. The difference between these two isolates is located in the N-terminal region. From previous research the N-terminal region of CP determines host-specific long distance movement for isolates of WSMV (Tatineni *et al.*, 2011). It was speculated perhaps this explained the conservation

of these two isolates within the TriMV population as a whole. The first replication of TriMV isolates in co-infected host plants with both TriMV isolates, suggested that TriMV_5262 might be better adapted to host plants such as wheat and rye. However, the results from replication 2 found both isolates equally adapted to the hosts examined. Our data therefore suggest that both TriMV isolates are equally adapted to infecting barley, rye, triticale, and wheat. It is interesting that these two TriMV isolates, with only a single nucleotide difference within their N-terminal region of the CP cistron, are highly conserved within the natural TriMV population. An evolutionary force, such as negative selection on the CP must be influencing the TriMV population as a whole within the various host species.

Since the TriMV isolates used for co-infection were not tagged with fluorescent proteins, it could not be determined if spatial exclusion occurred within the host's leaves. The results did demonstrate that one TriMV isolate did not fully inhibit the other from moving systemically, since both isolates were detected within a systematic leaf at least once in all four hosts. This is in agreement with previous co-infection studies of isolates of the same virus. A study done with WSMV and *Cucumber mosaic virus* detected both isolates within systemic leaves, although there were spatial exclusions (Hall *et al.,* 2001a; Takeshita *et al.,* 2004). Further research would be needed to determine if spatial segregation between these two TriMV isolates is occurring.

Conclusion

These findings help explain why TriMV_4685 and TriMV_5262 are conserved within the natural TriMV populations. With no significant differences in the CP mutation

frequency obtained from various host species within or between isolates, their base rate of diversity will be similar or identical. In addition, the lack of preference for one host over another suggests that each isolate is equally adapted to the four hosts examined. More research is needed to fully understand the various evolutionary factors working on plant RNA viruses within their host species.

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TABLES

Table III-1. Mutation frequency found in single TriMV_4685 inoculations. Plants number one through five are from replication 1, while plants number six through ten are from replication 2.

	Barley			**Rye**		
Plant #	No. of polymorphic sites/no. of nt	Mutation frequency/nt	Corrected total*	No. of polymorphic sites/no. of nt	Mutation frequency/nt	Corrected total*
1	0	-	-	2	9.950×10^{-4}	9.362×10^{-4}
2	2	9.950×10^{-4}	9.362×10^{-4}	0	-	-
3	0	-	-	2	9.950×10^{-4}	9.362×10^{-4}
4	2	9.950×10^{-4}	9.362×10^{-4}	0	-	-
5	3	14.925×10^{-4}	14.337×10^{-4}	1	4.975×10^{-4}	4.387×10^{-4}
6	0	-	-	0	-	-
7	0	-	-	0	-	-
8	0	-	-	0	-	-
9	0	-	-	0	-	-
10	0	-	-	0	-	-
	7/40200	1.741×10^{-4}	1.153×10^{-4}	5/40200	1.244×10^{-4}	0.656×10^{-4}
	Triticale			**Wheat**		
Plant #	No. of polymorphic sites/no. of nt	Mutation frequency/nt	Corrected total*	No. of polymorphic sites/no. of nt	Mutation frequency/nt	Corrected total*
1	0	-	-	0	-	-
2	0	-	-	1	4.975×10^{-4}	4.387×10^{-4}
3	0	-	-	0	-	-
4	3	14.925×10^{-4}	14.337×10^{-4}	1	4.975×10^{-4}	4.387×10^{-4}
5	1	4.975×10^{-4}	4.387×10^{-4}	0	-	-
6	0	-	-	0	-	-
7	1	4.975×10^{-4}	4.387×10^{-4}	0	-	-
8	0	-	-	1	4.975×10^{-4}	4.387×10^{-4}
9	0	-	-	0	-	-
10	0	-	-	0	-	-
	5/40200	1.244×10^{-4}	0.656×10^{-4}	3/40200	0.75×10^{-4}	0.162×10^{-4}

* Changes due to RT-PCR error for CP, RT error (0.588 x 10-4/nt) and PCR 40 cycles using *Pfu* II Ultra (0 /nt)

Table III-2. Mutation frequency found in single TriMV_5262 inoculations. Plants number one through five are from replication 1, plants number six through ten are from replication 2.

	Barley			**Rye**		
Plant #	No. of polymorphic sites/no. of nt	Mutation frequency/nt	Corrected total*	No. of polymorphic sites/no. of nt	Mutation frequency/nt	Corrected total*
1	0	-	-	0	-	-
2	0	-	-	0	-	-
3	0	-	-	1	4.975×10^{-4}	4.387×10^{-4}
4	2	9.950×10^{-4}	9.362×10^{-4}	1	4.975×10^{-4}	4.387×10^{-4}
5	1	4.975×10^{-4}	4.387×10^{-4}	1	4.975×10^{-4}	4.387×10^{-4}
6	0	-	-	0	-	-
7	0	-	-	0	-	-
8	0	-	-	0	-	-
9	1	4.975×10^{-4}	4.387×10^{-4}	0	-	-
10	1	4.975×10^{-4}	4.387×10^{-4}	1	4.975×10^{-4}	4.387×10^{-4}
	5/40200	1.244×10^{-4}	0.656×10^{-4}	4/40200	0.995×10^{-4}	0.407×10^{-4}

	Triticale			**Wheat**		
Plant #	No. of polymorphic sites/no. of nt	Mutation frequency/nt	Corrected total*	No. of polymorphic sites/no. of nt	Mutation frequency/nt	Corrected total*
1	0	-	-	0	-	-
2	0	-	-	0	-	-
3	0	-	-	1	4.975×10^{-4}	4.387×10^{-4}
4	0	-	-	0	-	-
5	3	14.925×10^{-4}	14.337×10^{-4}	1	4.975×10^{-4}	4.387×10^{-4}
6	0	-	-	0	-	-
7	1	4.975×10^{-4}	4.387×10^{-4}	0	-	-
8	0	-	-	1	4.975×10^{-4}	4.387×10^{-4}
9	0	-	-	2	9.950×10^{-4}	9.362×10^{-4}
10	0	-	-	0	-	-
	4/40200	0.995×10^{-4}	0.407×10^{-4}	5/40200	1.244×10^{-4}	0.656×10^{-4}

* Changes due to RT-PCR error for CP, RT error (0.588×10^{-4}/nt) and PCR 40 cycles using *Pfu* II Ultra (0 /nt)

Table III-3. Nucleotide substitutions detected within TriMV_4685 inoculated barley, rye, triticale, and wheat. Non-synomous mutations are denoted in bold. Samples were labeled with a B for barley, R for rye, T for triticale, and W for wheat, followed by the plant number from which it was obtained, and the clone number.

	TriMV_4685		
Host	**Plant / Clone**	**Mutation**	**Amino Acid Change**
Barley	B-2-8	$C_{9151} \rightarrow U$	Asp → Asp
		$G_{9478} \rightarrow A$	**Glu → Lys**
	B-2-9	$C_{9151} \rightarrow U$	Asp → Asp
		$G_{9478} \rightarrow A$	**Glu → Lys**
	B-4-2	$C_{9151} \rightarrow U$	Asp → Asp
		$G_{9478} \rightarrow A$	**Glu → Lys**
	B-4-7	$C_{9151} \rightarrow U$	Asp → Asp
		$G_{9478} \rightarrow A$	**Glu → Lys**
	B-8-4	$G_{9506} \rightarrow A$	**Ala → Thr**
	B-8-6	$C_{9723} \rightarrow U$	**Ala → Val**
		$U_{9963} \rightarrow :$	**deletion**
Rye	R-1-5	$C_{9151} \rightarrow U$	Asp → Asp
		$G_{9478} \rightarrow A$	**Glu → Lys**
	R-1-9	$C_{9151} \rightarrow U$	Asp → Asp
		$G_{9478} \rightarrow A$	**Glu → Lys**
	R-3-5	$C_{9151} \rightarrow U$	Asp → Asp
		$G_{9478} \rightarrow A$	**Glu → Lys**
	R-8-3	$G_{10037} \rightarrow U$	**Gly → Cys**
Triticale	T-4-4	$C_{9151} \rightarrow U$	Asp → Asp
	T-4-5	$C_{9806} \rightarrow A$	**Pro → Thr**
		$A_{9822} \rightarrow G$	**Asn → Ser**
	T-5-1	$A_{9822} \rightarrow G$	**Asn → Ser**
	T-7-2	$U_{9755} \rightarrow C$	**Phe → Leu**
Wheat	W-2-1	$C_{9768} \rightarrow U$	**Ala → Val**
	W-4-1	$C_{9795} \rightarrow U$	**Ala → Val**
	W-8-1	$U_{9745} \rightarrow C$	Val → Val

Table III-4. Nucleotide substitutions detected within single TriMV_5262 inoculations on various hosts. Non-synomous mutations are denoted in bold. Samples were labeled with a B for barley, R for rye, T for triticale, and W for wheat, followed by the plant number from which it was obtained, and the clone number.

	TriMV_5262		
Host	**Plant / Clone**	**Mutation**	**Amino Acid Change**
Barley	B-7-1	$T_{9182} \rightarrow C$	**Phe → Leu**
		$G_{9799} \rightarrow A$	Lys → Lys
	B-18-2	$T_{9877} \rightarrow C$	Phe → Phe
	B-19-2	$T_{9858} \rightarrow C$	**Phe → Ser**
	B-20-1	$G_{9801} \rightarrow A$	**Arg → His**
Rye	R-3-3	$T_{9744} \rightarrow C$	**Val → Ala**
	R-7-2	$T_{9572} \rightarrow C$	**Trp → Arg**
	R-8-2	$C_{9190} \rightarrow G$	**Phe → Leu**
	R-9-4	$G_{9040} \rightarrow A$	Gly → Gly
Triticale	T-6-2	$T_{9433} \rightarrow C$	Asp → Asp
		$C_{9591} \rightarrow :$	**deletion**
		$A_{9969} \rightarrow T$	**Lys → Met**
	T-7-2	$A_{9084} \rightarrow C$	**Glu → Ala**
Wheat	W-3-2	$G_{9716} \rightarrow :$	**deletion**
	W-5-1	$T_{9555} \rightarrow G$	**Val → Gly**
	W-6-1	$G_{9926} \rightarrow T$	**Val → Phe**
	W-7-1	$C_{9151} \rightarrow T$	Asp → Asp
	W-7-2	$T_{9211} \rightarrow C$	Ser → Ser

Table III-5. Double inoculation experiment conducted using TriMV_4685 and TriMV_5262 on four different host plants. Five plants were collected per host, obtaining two TriMV clones from each upper systemic leaf sample. Plants with both isolates detected are denoted in the third column.

	Replication 1			**Replication 2**		
	TriMV_4685	TriMV_5262	Plants with both isolates	TriMV_4685	TriMV_5262	Plants with both isolates
Barley	5	5	1	5	5	1
Rye	1	9	1	5	5	1
Triticale	6	4	0	7	3	1
Wheat	3	7	3	5	5	1

FIGURES

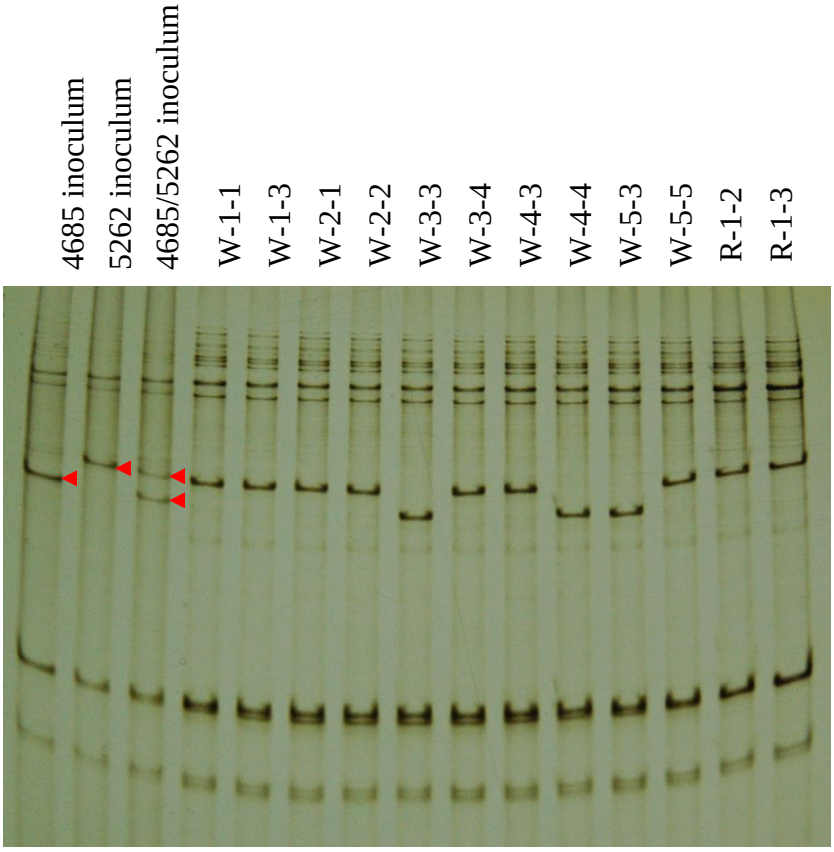

Figure III-1. TriMV coat protein (CP) cistrons analyzed by SSCP assay. PCR products denatured and digested with restriction enzymes were electrophoresed on 10% polyacrylamide at 20ºC and 4ºC under nondenaturing conditions. SSCP patterns unlike the inoculum patterns at one or both temperatures corresponded to single-nucleotide substitution creating new haplotypes.

CHAPTER 4

Genetic Stability Within the P1 and CP Cistrons of *Triticum Mosaic Virus* Maintained After Horizontal Transfer by Wheat Curl Mites Through Alternative Hosts

ABSTRACT

Plant viral populations may be subjected to at least three bottlenecks that are understood to limit their genetic variation: vertical transmission, vector transmission, and systemic movement within the host plant. Few studies have been conducted to determine how bottlenecking severity, associated with horizontal transfer of plant RNA viruses affects viral diversity. A sequenced *Triticum mosaic virus* (TriMV) isolate was used to observe the variation of TriMV populations when horizontally transmitted by the wheat curl mite (*Aceria tosichella*). Wheat curl mites were used to transmit TriMV from wheat (*Triticum aestivum cv.* Millennium) to three alternative hosts, barnyardgrass (*Echinochloa crus-galli*), green foxtail (*Seteria viridis*), and jointed goatgrass (*Aegilops cylindrica*) and then back to wheat. Protein 1 (P1) and coat protein (CP) cistrons of TriMV were then analyzed using single-strand conformational polymorphism (SSCP) on viral genomes isolated from each host, to determine if there were changes in the viral sequence within these regions. Genetic stability of TriMV was maintained through genetic bottlenecking when horizontally transferred by wheat curl mites and systemic movement through wheat and jointed goatgrass.

INTRODUCTION

The short replication time and error-prone polymerase of RNA viruses have the potential to create large diverse populations (Holland *et al.,* 1982; Novella *et al.,* 1995; McNeil *et al.,* 1996; Hall *et al*., 2001; Harrison, 2002; French and Stanger, 2003; García-Arenal *et al.,* 2003; Elena *et al.,* 2008; Gao and Feldman, 2009; Tromas and Elena, 2010). Sequence diversity between plant RNA virus isolates has been established, although, little variation has been identified within individual populations (Kurath and Palukaitis, 1990; Albiach-Martí, *et al*., 2000; Kong *et al.,* 2000; French and Stenger, 2003; García-Arenal *et al.,* 2003). This leads to the question: what is keeping plant RNA virus populations from being vastly diverse? Viral genomes for (+)-sense, single-stranded RNA viruses are made in a predominately linear "stamping machine" model (French and Stenger, 2003). The "stamping machine" model describes how a few negative strand templates are made from a newly invading positive strand which are then used to make several thousand positive strands in a stamping machine manner. New mutations arising during negative strand production are far more likely to be amplified than new mutations generated in the progeny (positive strands). Mutants must be replicated and able to move within the host to increase the virus population's diversity.

Negative selection and genetic bottlenecking are evolutionary factors that affect many plant viruses, suggesting these forces must play a role in limiting genetic variation (Rodriguez-Cerezo *et al.,* 1991; Choi *et al.,* 2001; Hall *et al.,* 2001; Lin *et al.,* 2003; Sacristán *et al.,* 2003; García-Arenal *et al.,* 2003; Li and Roossinck, 2004; French and Stenger, 2005; Ali *et al.,* 2006; Elena *et al.,* 2008; Acosta-Leal *et al.,* 2011). There are at

least three bottlenecks understood to limit the genetic variation in plant viral populations: vertical transmission (by seed), vector transmission, and systemic movement within the host plant (Sacristán *et al.,* 2003; Li and Roossinck, 2004; Elena *et al.,* 2008; Simmons *et al.,* 2013). Severe bottlenecking events have been observed during systemic movement, with merely five to eleven virus genomes out of 10^6 moving to an adjacent cell to initiate a new infection, depending on the plant virus (French and Stanger, 2005; Ali *et al.,* 2006; González-Jara *et al.,* 2009; Miyashita and Kishino, 2010; Gutiérrez *et al.,* 2010; Bartels, 2011). Few studies have determined the severity of bottlenecking that may occur during horizontal transfer of plant RNA viruses. Among those that have been completed, one study revealed only one to three *Potato virus Y* particles were transmitted by an aphid to initiate a successful infection (Moury *et al.,* 2007). Similar results were found for *Cucumber mosaic virus,* with one to two particles transferred by an aphid (Betancourt, *et al.,* 2008).

Within the Great Plains region, field samples of *Triticum mosaic virus* (TriMV) isolates were established to be homogenous (Fuentes-Bueno *et al.,* 2011). The variation of TriMV when horizontally transmitted by the wheat curl mite (WCM), (*Aceria tosichella*), was addressed in this research. A Nebraska TriMV isolate was used for WCM transmission of TriMV from wheat (*Triticum aestivum cv.* Millennium) to three alternative hosts: barnyardgrass (*Echinochloa crus-galli*), green foxtail (*Seteria viridis*), and jointed goatgrass (*Aegilops cylindrical*), then back to wheat. The protein 1 (P1) and coat protein (CP) cistrons of TriMV populations were analyzed from each host to determine if a drastic shift in sequence variation was observed. Variation was determined by single-strand conformational polymorphism (SSCP).

MATERIAL AND METHODS

Source wheat plants

Three seeds of wheat were planted in each of the 28 cone-tainers filled with pre-mixed sterilized potting medium. Plastic mite-secure cages with Nytex® mesh (250-micron mesh; BioQuip Products Inc.) screens were placed over each cone-tainer (McMechan, 2012; McMechan *et al.,* 2014). Plants were grown in a greenhouse maintained at 20˚C to 26˚C and watered from the bottom by plastic tubs. At eight days after planting, wheat plants were thinned to one plant per cone-tainer.

TriMV inoculation of source wheat plants

Fourteen wheat plants were inoculated with Nebraska TriMV isolate N10_4685 at 11 days after planting (Nebraska strain GenBank, accession no. FJ669487) (Tatineni *et al.,* 2009). A 1:10 dilution of inoculum was prepared by grinding two grams of frozen N10_4685 infected tissue (chapter 3) in 20 ml of sterile water using a mortar and pestle. Five hundred µl of inoculum was collected in a microfuge tube to be used as a positive control (founding inoculum) for SSCP analysis. Carborundum dusted plants were inoculated mechanically by rubbing inoculum onto the leaves using gloved finger tips. Twelve wheat plants were mock-inoculated with sterile water as a control.

Establishment of viruliferous mite population

At five days post inoculation, wheat plants inoculated with TriMV sap or water (mock inoculated) were infested with ten non-viruliferous wheat curl mites (WCM).

Nebraska Type 2 WCM were utilized in this experiment because they have been found to be the most efficient in transmitting TriMV (Hein *et al.,* 2012; McMechan *et al.,* 2014). Only adults and nymphs were transferred to wheat source plants. WCM populations were maintained and transferred as described by McMechan *et al.* (2014). Ten WCM were placed onto a black triangle and placed in the axial of the plant under a dissecting microscope. After each infestation, plants remained in the lab for a period of 10 to 15 hours to enable WCM to settle on the plants. All mite infested pots were transferred to a growth chamber with a 14:10 (L:D) light cycle maintained at 27°C. All 24 wheat source plants were collected for RT-PCR analysis.

Alternative host plants

The alternative host, barnyardgrass, was planted the same day as the wheat source plants were infested with WCM. Three six-inch diameter pots were seeded with approximately 200 barnyardgrass seeds. Seven days after infesting wheat source plants with WCM, the remaining test plants were seeded in six-inch diameter pots. Three pots were seeded with approximately ten and 200 seeds of jointed goatgrass and green foxtail, respectively. Three pots were each seeded with ten wheat seeds. Each host pot was thinned to eight plants prior to WCM infestation. Pots were covered with plastic mite secure cages with Nytex® mesh (250-micron mesh; BioQuip Products Inc.).

WCM infestation of alternative hosts and sampling

At 14 days after planting, pots containing alternative hosts and the primary host (wheat) were infested with ten WCM each, taken from a random wheat source plant

chosen by randomization using SAS software version 9.2 (SAS Institute Inc.). For each alternative host (barnyardgrass, jointed goatgrass, and green foxtail) and wheat, TriMV virulent WCM were infested in replicates of three pots per host. One plant was harvested from each pot at 7, 14, 21, 28, 35, and 42 days post infestation (dpi) of WCM. Except for wheat, time points were 7, 14, and 21 day post infestation. Samples were combined to form three time points 14, 28, and 42 dpi. Therefore, plants harvested at 7 and 14 dpi were combined for RT-PCR and single-strand conformational polymorphism (SSCP) analysis.

Virulent WCM infestation of wheat plants

Twelve six-inch diameter pots were planted with ten wheat seeds at 35 dpi of WCM on the alternative hosts. At 49 dpi, ten WCM were transferred from an alternative host plant to a pot of wheat plants at 14 days old. One wheat plant was harvested from each pot at 7, 14, and 21 dpi. The three time points were combined into one sample for RT-PCR and SSCP analysis.

Reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA isolated from tissue samples obtained at the various time points was stored at -80ºC (see chapter 2 “Total RNA extraction”). RT-PCR was performed on total RNA isolated from each sample using P1 and CP specific primers. Primers targeting part of the 5’UTR and the whole P1 cistron of TriMV consisted of a forward primer Tr-72 annealing to nucleotide position 301 to 318, (5’-GATGAGCTCTACAAATAAGGGCTTAGGCGATTGTAC-3’) and a reverse

primer Tr-76 annealing to nucleotide position 1,860 to 1,888, (5'-GAGAGAGCTCCTAGTAATATGTCAAGCCCTCTAAGCAATCAG -3'). Primers annealing to the CP region of TriMV were comprised of a forward primer Tr-89 annealing to nucleotide position 9,001 to 9,019, (5'-CAAGATTAACGCGGCATGG-3') and a reverse primer Tr-100 annealing to nucleotide position 10,048 to 10,075, (5'-AACCTCGAGCTAACGGGTACCAAACATGGCCCCGCCGACA-3').

Reverse transcription was performed on total RNA samples at 42˚C for 1 hour, followed by 99˚C for 5 min and held at 10˚C. Each reverse transcription reaction consisted of 5.9 µl of sterile water, 2 µl of 5x buffer (RT AMV), 0.5 µl of 50 ng/µl virus specific reverse primer, 0.4 µl of 10 mM dNTPs, 0.2 µl of RT AMV (Roche, Indianapolis, IN) (23 U/µl) and 1 µl total RNA isolated from systemic leaves. Two PCR reactions were performed on a sample targeting the P1 or CP regions, using high fidelity *Pfu* II Ultra polymerase (Agilent Technologies) (Figure II-1). One µl cDNA was used for PCR in a 25 µl reaction volume with P1 or CP specific primers. The PCR program consisted of: 95˚C for 2 min, followed by 10 cycles at 95°C for 30 s, 48˚C for 30 s and 72˚C for 2 min. The annealing temperature was raised to 56˚C for the remaining 30 cycles. The PCR program consisted of 40 cycles on a T1 thermocycler (Biometra). All reactions were incubated at 25˚C, removed from the thermocycler, and stored at -20˚C. The RT-PCR products were analyzed on 1.0% agarose gels in TBE (Tris-borate-EDTA) buffer.

Single-strand conformation polymorphism (SSCP) analysis

SSCP analysis was performed on samples obtained from wheat source plants, wheat and jointed goatgrass test plants and back to wheat for both the P1 and CP regions. The final PCR products were digested with the appropriate restriction enzymes into ~200 to 500 bp fragments required for SSCP analysis (Orita *et al.,* 1989). Three restriction enzymes, *Ban* I, *Pst* I and *Pvu* II were utilized to digest P1 PCR products into four fragments. Restriction enzyme *Dde* I digested the CP PCR product into four fragments. Digested samples were prepared for SSCP as described in Hall *et al.,* (2001). Ten percent Tris/Borate/EDTA (TBE) polyacrylamide gels (Life Technologies, Carlsbad, CA) were electrophoresed in 1X TBE buffer at 20ºC and 4ºC at 60 volts for 16 ½ hours and 45 volts for 15 ½ hours, respectively. This was done because the accuracy of SSCP to detect single nucleotide polymorphisms improves to ~98% when two gels are analyzed at alternate temperatures (Welsh *et al.,* 1997).

Silver staining of SSCP gels was performed using a GE Healthcare DNA Silver Staining Kit PlusOne (Waukesha, WI), and following the protocol provided (Pawlotsky *et al.,* 1998). Samples displaying differential SSCP patterns from that of the positive control (plasmid TriMV) were further selected for nucleotide sequencing. The miniprep DNAs (30 µl) were resuspended in 170 µl of Tris-EDTA buffer (pH 8) and treated with 2 µl RNase A (1ng/µl) for 20 minutes at 37ºC, and then extracted with phenol:chloroform (see Chapter 2 "Plasmids prepared for sequencing"). Samples were sent for sequencing to the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida. Sequence results were analyzed by Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI).

RESULTS AND DISCUSSION

Verification of source and test plants infected with TriMV

Wheat source plants inoculated with TriMV displayed symptoms such as mosaic and chlorotic spotting on the leaves at 14 days post inoculation. The presence of TriMV in each wheat source plant was verified by RT-PCR amplification of the CP region. All 12 mechanically inoculated source plants tested positive for TriMV, and the two mock inoculated source plants testing negative (Figure IV-1).

TriMV symptoms were not observed on barnyardgrass infested with TriMV virulent mites (Figure IV-2). Barnyardgrass plants in all three pots at time points 14, 28, and 42 dpi tested negative for the presence of TriMV (Table IV-1 and Figure IV-3). Barnyardgrass has been reported as a nonhost for TriMV in a study done by Seifers *et al.*, (2010), where researchers mechanically inoculated a Kansas isolate of TriMV onto 27 barnyardgrass plants, with no symptomatic plants observed. However, a recent finding detected TriMV within field collected barnyardgrass harvested from western Nebraska, High Plains Agricultural Lab (McMechan, unpublished). There are several possible explanations for this discrepancy. One could be that the Nebraska Type 2 WCM used in this research was not able to transmit TriMV to barnyardgrass. The WCM population observed on barnyardgrass should have been adequate for TriMV inoculation. Barnyardgrass in this study was particularly small compared to barnyardgrass found in a field setting, possibly due to artificial lighting or suboptimal day length in the growth chamber (Figure IV-2). Other factors that could have resulted in a lack of TriMV

infection in barnyardgrass might be stage-related to the plants growth or improper timeframe of mite presences on the host.

For the alternative host, green foxtail, TriMV symptoms were not observed on any of the plants infested with TriMV virulent mites (Figure IV-2). Samples from green foxtail at 14, 28, and 42 dpi, with three pots each tested negative for TriMV by RT-PCR (Table IV-1 and Figure IV-3). Green foxtail is known to be an alternative host of TriMV (Seifers *et al.*, 2010). The green foxtail plants grown for this experiment were stunted, possibly due to artificial lighting or unsuitable day length in the growth chamber (Figure IV-2). TriMV was likely not transmitted, due to WCM populations not thriving on green foxtail. For the majority of the green foxtail plants, no WCM were observed at the given time points. Jointed goatgrass, an alternate host of TriMV and wheat, a primary host of TriMV, both displayed mosaic symptoms on their leaves throughout the study (Figure IV-2). The presence of TriMV was verified in all samples of jointed goatgrass and wheat by RT-PCR amplification of the CP region (Figure IV-3).

TriMV viruliferous WCM transferred from alternative hosts to wheat

Wheat plants infested with WCM from alternative hosts were tested for TriMV infection and the results are presented in Table IV-1 at 7 to 21 dpi. Wheat infested with WCM from wheat test plants tested positive for the presence of TriMV (Table IV-1). For green foxtail at 49 dpi, no live mites could be found; therefore mites could not be transferred back to wheat (Table IV-1). Wheat infected with virulent WCM from jointed goatgrass tested positive for the presence of TriMV (Table IV-1). Wheat plants infested with WCM transferred from barnyardgrass remained uninfected by TriMV (Table IV-1).

This was expected, because TriMV was not detected in barnyardgrass at the previous time points; consequently there was no TriMV for the WCM to horizontally transfer back to wheat plants.

SSCP and sequencing results

Only samples where TriMV infection was detected in the wheat source plant, the test plant and in the final wheat plant were analyzed by SSCP. By using the WCM, we hoped to observe shifts in the population of TriMV CP haplotypes since, severe bottlenecking events occur during horizontal transmission. SSCP patterns unlike the founding inoculum of TriMV isolate 4685 (Nebraska strain) would indicate a single-nucleotide substitution within the sample. No polymorphisms were discovered within the P1 region of sample sets obtained from wheat (Figure IV-4) or jointed goatgrass (Figure IV-5).

Polymorphisms were observed within the CP region of samples extracted from wheat in pot 1, at 14, 28, and 21 dpi (Figure IV-6). For pot 2 and 3 of wheat, polymorphisms were observed at 14 and 28 dpi, respectively (Figure IV-6). Within the CP region of samples extracted from jointed goatgrass, polymorphisms were detected in pot 1, 2, and 3 at all four time points observed (Figure IV-7). Samples established by SSCP analysis to have polymorphisms were sequenced (Table IV-2). The sequence results revealed no true polymorphisms in any of the samples sequenced (Table IV-2). The bands displayed on the SSCP gels, unlike the positive control, were most likely due to contamination of the sample. Such as the CP specific primers might have mismatched to the WCM or possibly the host plant DNA.

By not cloning several viral genomes from each total RNA sample, the haplotype obtained was assumed to be the predominate haplotype within the whole viral population. Our results indicate virus populations do maintain genetic stability, even with genetic bottlenecking caused by horizontal transfer by WCM and systemic movement within alternative hosts. This is in agreement with previous studies which showed there was little variation within TriMV isolates obtained from field samples (Fuentes-Bueno *et al.*, 2011).

We can concluded from this research that passage in the alternative host, jointed goatgrass, caused no detectable shift away from the founding haplotype CP sequence within the TriMV populations observed. This suggests that bottlenecks occurring within jointed goatgrass are similar or identical to those happening within wheat. Our results indicate that virus populations can maintain genetic stability, even when there is genetic bottlenecking caused by horizontal transfer by WCM. More research is needed to address how many virus particles of TriMV are transmitted by a single WCM in order, to fully understand the severity of the bottlenecks that occurs during mite transmission of virus populations.

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TABLES

Table IV-1. TriMV infection in samples taken from the alternative hosts at three time points; days post infestation (dpi) of virulent wheat curl mites. Positives are represented with a plus, while those samples testing negative for TriMV are represented with a negative sign. An X indicates no sample was obtained at that time point for that particular host. TriMV presence was verified by amplifying the CP region using RT-PCR.

			TriMV Infection				
Host	**Trt**	**Pot**	**7-14 dpi**	**21-28 dpi**	**35-42 dpi**	**Back to Wheat**	**7-21 dpi**
Wheat	Mock	1	-	-	-	Wheat	-
Wheat	Mock	2	-	-	-	Wheat	-
Wheat	Mock	3	-	-	-	Wheat	-
Wheat	TriMV	1	+	+	X	Wheat	+
Wheat	TriMV	2	+	+	X	Wheat	+
Wheat	TriMV	3	+	+	X	Wheat	+
Green Foxtail	Mock	1	-	-	-	Wheat	X
Green Foxtail	Mock	2	-	-	-	Wheat	X
Green Foxtail	Mock	3	-	-	-	Wheat	X
Green Foxtail	TriMV	1	-	-	-	Wheat	X
Green Foxtail	TriMV	2	-	-	-	Wheat	X
Green Foxtail	TriMV	3	-	-	-	Wheat	X
Jointed Goatgrass	Mock	1	-	-	-	Wheat	-
Jointed Goatgrass	Mock	2	-	-	-	Wheat	-
Jointed Goatgrass	Mock	3	-	-	-	Wheat	-
Jointed Goatgrass	TriMV	1	+	+	+	Wheat	+
Jointed Goatgrass	TriMV	2	+	+	+	Wheat	+
Jointed Goatgrass	TriMV	3	+	+	+	Wheat	+
Barnyardgrass	Mock	1	-	-	-	Wheat	-
Barnyardgrass	Mock	2	-	-	-	Wheat	-
Barnyardgrass	Mock	3	-	-	-	Wheat	X
Barnyardgrass	TriMV	1	-	-	-	Wheat	-
Barnyardgrass	TriMV	2	-	-	-	Wheat	-
Barnyardgrass	TriMV	3	-	-	-	Wheat	-

Table IV-2. Samples testing positive for polymorphisms by SSCP analysis were sequenced. No true mutations were revealed by sequence results.

Host	Pot	Days post infestation	Mutations Verified
wheat	1	14	0
		28	0
		21	0
wheat	2	14	0
		28	0
wheat	3	14	0
		28	0
jointed goatgrass	1	14	0
		28	0
		42	0
jointed goatgrass	2	14	0
		28	0
		42	0
jointed goatgrass	3	14	0
		28	0
		42	0

FIGURE

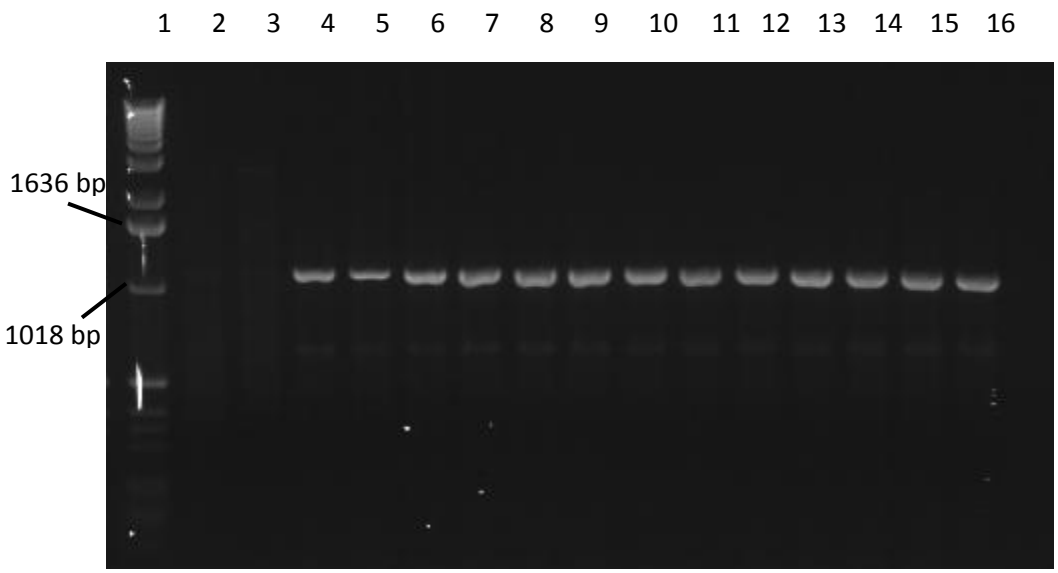

Figure IV-1. Verification of TriMV presence in wheat source plants by RT-PCR amplification of the CP. The CP fragment produced resulted in a 1,087 bp fragment. Lane 1 is a 1 kbp ladder, followed by mock source plants 1 and 2. Lanes 4 through 16 are wheat source plants # 1 through 12.

Wheat

Jointed Goatgrass

Green Foxtail

Barnyardgrass

Figure IV-2. Alternative host plants at 21 days post-infestation, displaying possible TriMV symptoms and plant growth.

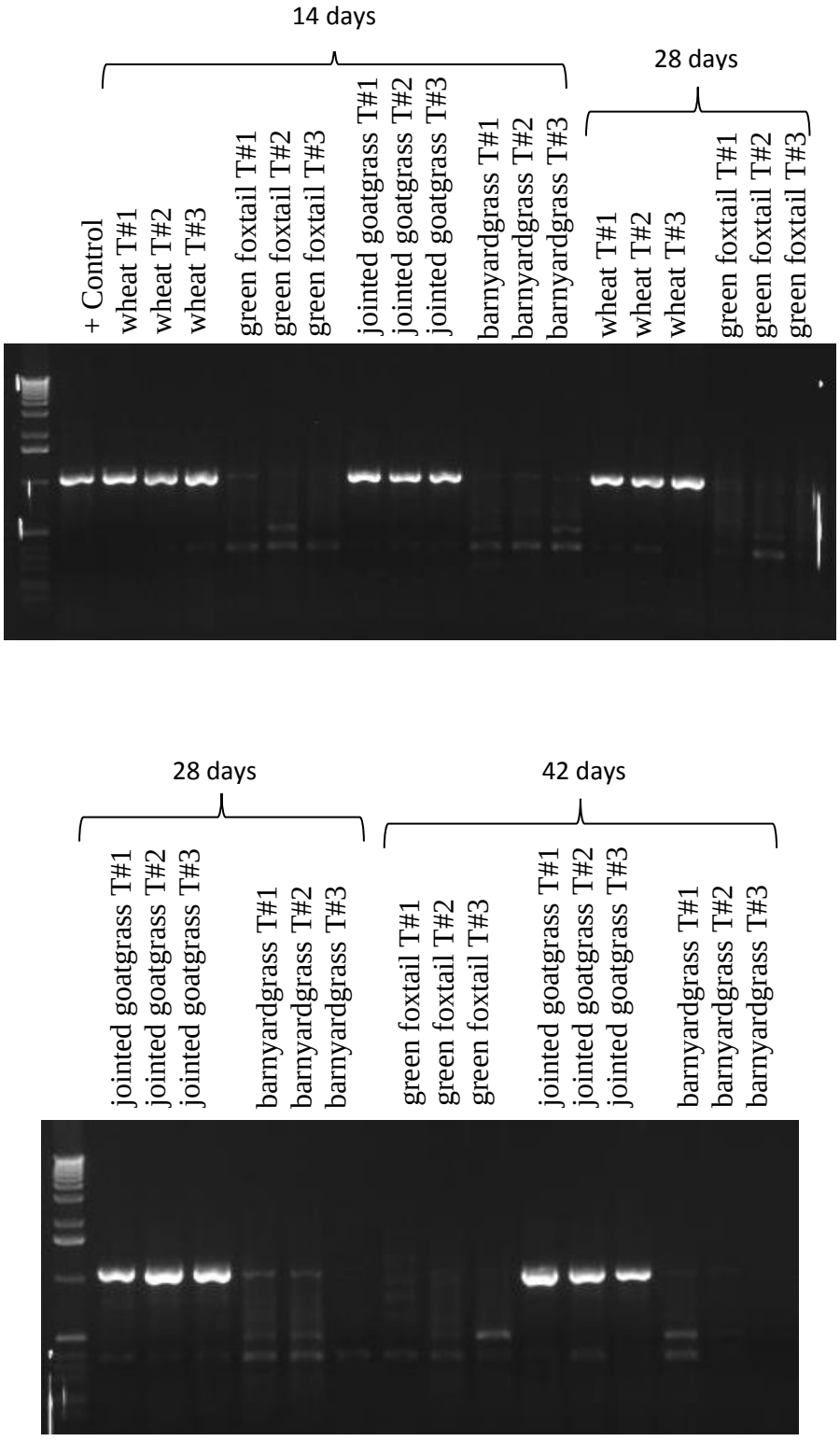

Figure IV-3. Verification of TriMV presence in test plants by targeting the CP using RT-PCR. The CP fragment produced resulted in a 1,087 nucleotide fragment. Lane 1 in each gel is a 1 kbp ladder. The lanes are labeled according to the PCR sample loaded. The positive control, TriMV isolate 4685, was used to inoculate wheat source plants.

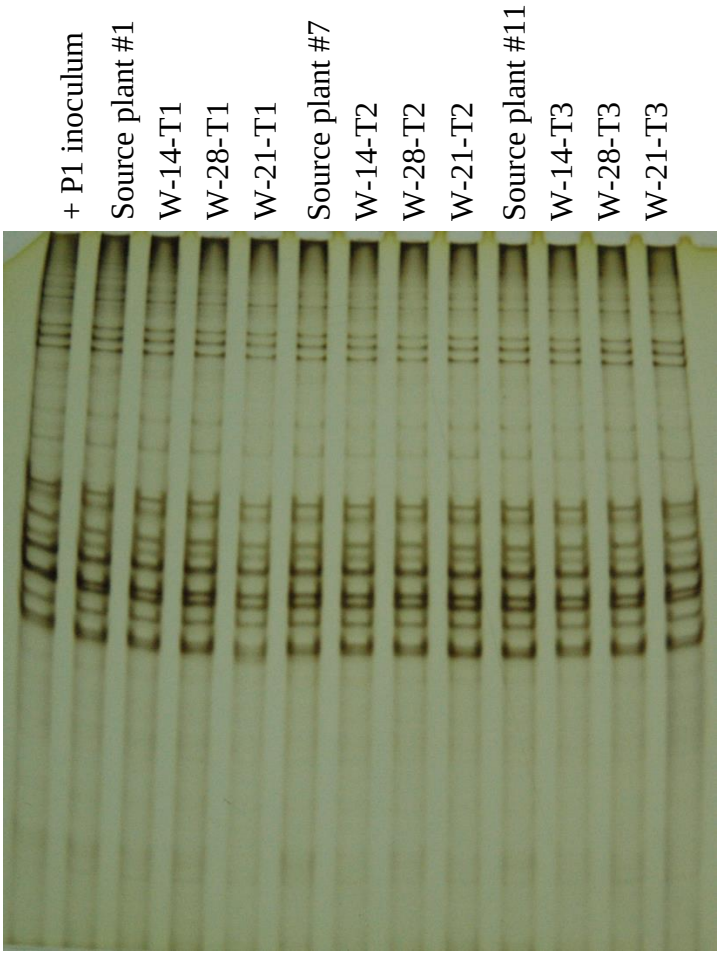

Figure IV-4. TriMV protein 1 (P1) obtained from wheat and analyzed by SSCP at 20ºC are shown. W-14-T1 stands for wheat plant, collected at 14 days post-infestation, replication 1 with TriMV. PCR products denatured and digested with restriction enzymes were electrophoresed on 10% polyacrylamide gels at 20ºC and 4ºC under nondenaturing conditions. SSCP patterns, unlike the inoculum pattern in lane 1 at one or both temperatures, corresponded to a single-nucleotide substitution.

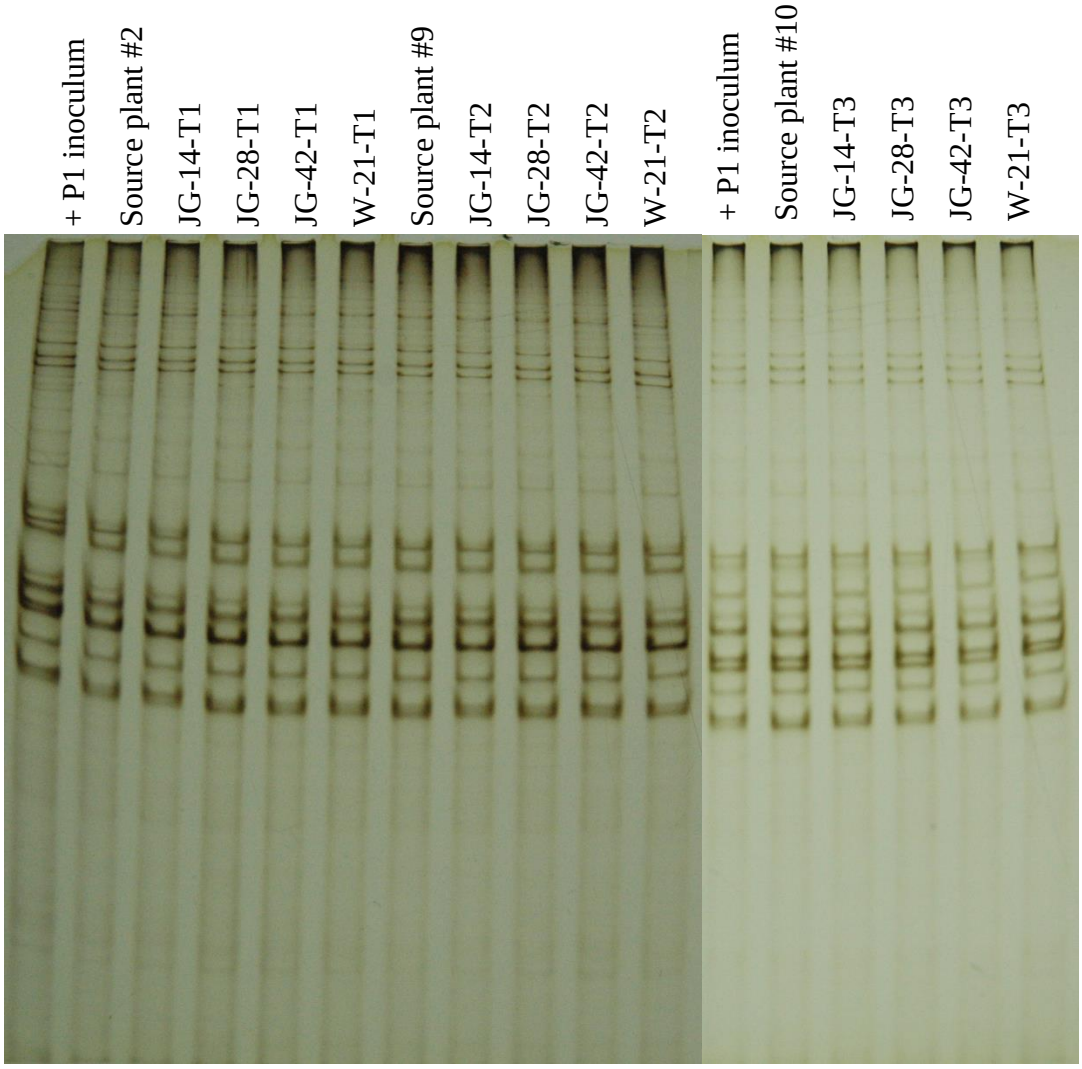

Figure IV-5. TriMV protein 1 (P1) extracted from jointed goatgrass and analyzed by SSCP gels at 20ºC are shown. JG-14-T1 stands for jointed goatgrass collected 14 days post-infestation from TriMV replication 1. SSCP patterns unlike the inoculum pattern at one or both temperatures corresponded to a single-nucleotide substitution.

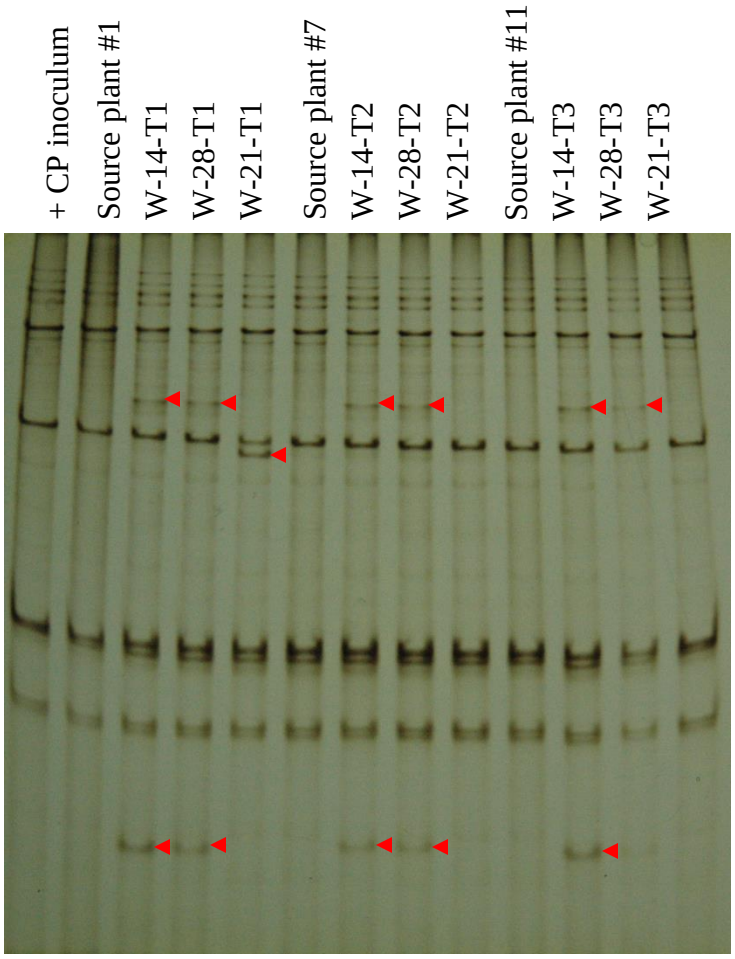

Figure IV-6. TriMV coat protein (CP) obtained from wheat and analyzed by SSCP gels at 20ºC are shown. W-14-T1 stands for wheat plant, collected at 14 days post-infestation, replication 1 with TriMV. SSCP patterns different from the control inoculum pattern in lane 1 are indicated by red arrows and correspond to that sample having a single-nucleotide polymorphism.

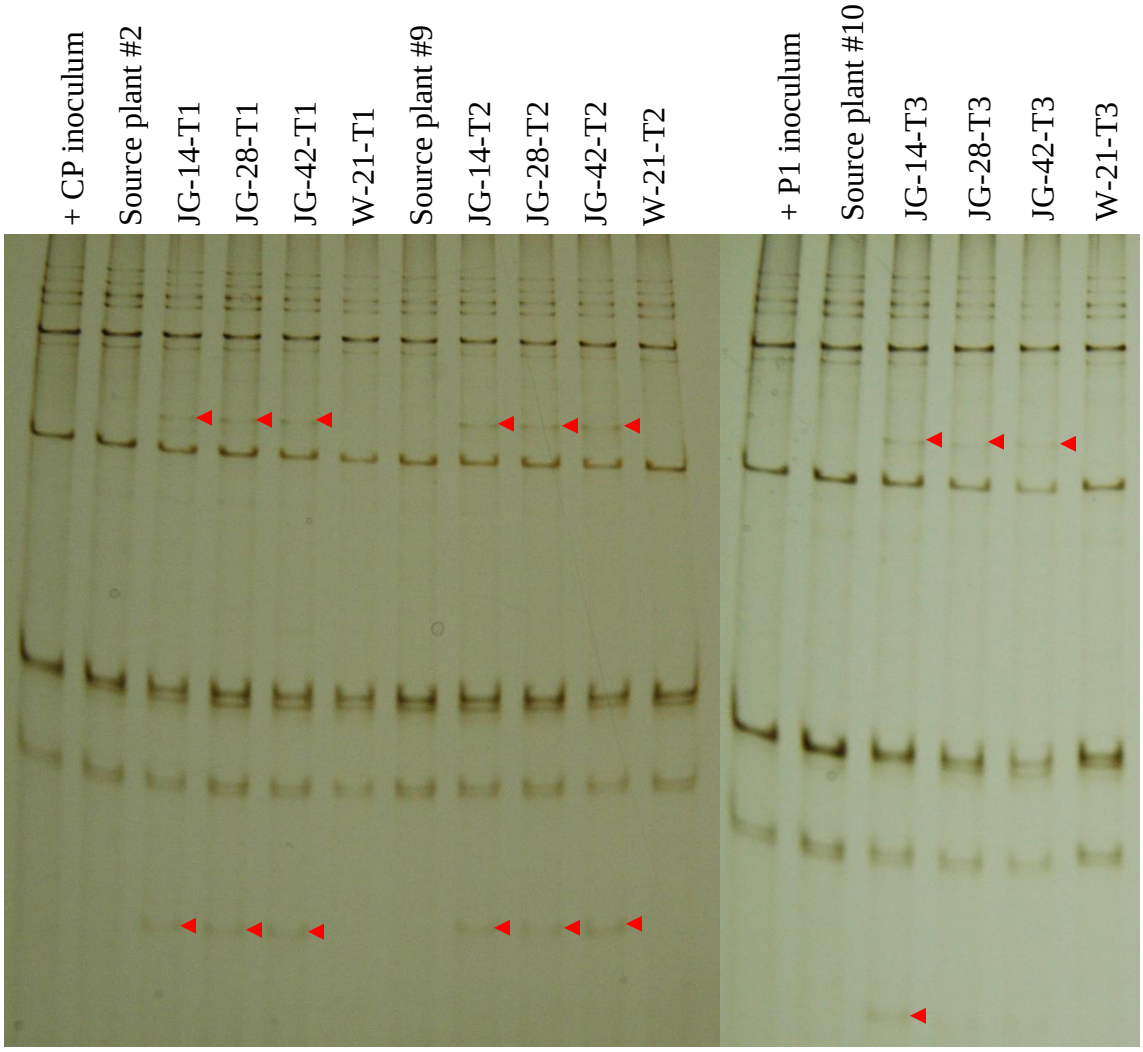

Figure IV-7. TriMV coat protein (CP) extracted from jointed goatgrass plants and analyzed by SSCP gels at 20ºC are shown. The following label, JG-14-T1 stands for jointed goatgrass plant, collected at 14 days post-infestation, and pot number 1 infected with TriMV. SSCP patterns different from the control inoculum pattern in lane 1 are indicated by red arrows and correspond to that sample having a single-nucleotide polymorphism.

CHAPTER 5

Concluding Remarks

It has been well established that RNA viruses should be genetically diverse, due to the high error rate of RNA-dependent RNA polymerases (approximately 10^{-4}/nt per replication), and the lack of proof-reading capabilities (Halls *et al.,* 2001; Harrison, 2002) are not as genetically diverse as expected. Therefore, evolutionary factors, such as purifying selection and genetic bottlenecks that favor genetic stability, might be affecting plant viral populations (Rodriguez-Cerezo *et al.,* 1991; Hall *et al.,* 2001; Schneider and Roossinck 2001; Li and Roossinck *et al.,* 2004; French and Stenger, 2005; Acosta-Leal *et al.,* 2011). Otherwise, RNA virus populations, with their potential for extreme diversity, might acquire a lethal number of mutations leading to collapse of the population. In this dissertation, I have demonstrated that these evolutionary factors are favoring genetic stability in TriMV populations grown in a controlled greenhouse environment.

Initially, we discovered that the mutation frequency per nucleotide of the TriMV P1 and CP cistrons was half that of similar plant viruses that have been examined, such as WSMV. The mutation frequencies we detected were lower, not only compared to other *Potyviridae* family members, but also compared to (+)-sense, single stranded plant RNA viruses in general. The TriMV population variation observed after serial passage were dominated by singletons within both the P1 and CP cistrons. Thus, stochastic processes such as bottlenecking must have impacted the observed populations. This is consistent with past research which found that stochastic processes influenced other plant viruses (Choi *et al.,* 2001; Hall *et al.,* 2001; Lin *et al.,* 2003; Sacristán *et al.,* 2003; Li and Roossinck, 2004; French and Stenger, 2005; Ali *et al.,* 2006; Elena *et al.,* 2008; Acosta-Leal *et al.,* 2011). The founding inoculum type sequence was maintained at an

observable frequency throughout the serial passages and repeated bottlenecking events, supporting the conclusion that some level of genetic stability was being maintained.

Additionally, we demonstrated that the mutation frequencies observed within the CP of two TriMV strains within changing environments, such as passage in different host species, were not significantly different. Therefore, the base level of TriMV population diversity was maintained across different host species. This could mean that the evolutionary factors such as genetic bottlenecking, selection, and/or genetic drift working on the viral population are similar or the same within wheat, barley, rye, and triticale. In addition, the lack of preference for any particular host suggests that both TriMV strains are equally adapted to the four hosts examined.

Finally, the variation of the P1 and CP regions of TriMV populations, horizontally transmitted by the wheat curl mite, was determined. TriMV population genetic stability was also maintained over the course of this three month experiment, even during the added bottlenecking introduced during wheat curl mite transfer and systemic movement within the plants. The alternative host, jointed goatgrass, caused no drastic shift in the founding haplotype of CP within the TriMV populations observed, indicating that the bottlenecks occurring within this host are similar to, or the same as within wheat. Our results indicate TriMV populations do maintain genetic stability, even with genetic bottlenecking by horizontal transfer by wheat curl mites and systemic movement.

The TriMV populations observed maintained genetic stability by the evolutionary forces of selection and genetic bottlenecking. This leads us to conclude that selection and drift are not exclusive, and may occur concurrently within a virus population (García-Arenal *et al.*, 2003; French and Stenger, 2005; Ali *et al.*, 2006). The process of genetic

drift is fundamentally random, with the outcome within any single TriMV population not predetermined. This research shows that the combined effect of both forces acting simultaneously on different regions of the genome ultimately regulates the degree of sequence variation within virus populations. Bottlenecking leading to genetic drift can cause drastic virus population shifts, although this work has shown bottlenecking can also work to maintain the genetic stability of virus populations. The results obtained in this research are consistent with recent studies which have observed that TriMV isolates from the Great Plains region field samples are generally homogenous with little variation (Fuentes-Bueno *et al.,* 2011; Seifers *et al.,* 2013).

Basic knowledge of plant virus evolution is critical to the development of efficient management strategies, because resistance genes have been overcome by the evolution of pathogen populations. For example, two amino acids changes in the movement proteins of *Tobacco mosaic virus* or *Tomato mosaic virus* were capable of overcoming the resistance conferred by the *Tm-2* gene in tomatoes (Meshi *et al.,* 1989; Weber *et al.,* 1993). Researchers have just begun to uncover the evolutionary factors influencing plant RNA virus populations. More research is needed to discover the number of TriMV genomes which move from cell-to-cell and systemically within different host species. This would allow researchers to determine the severity of bottlenecking occurring within each host species. It will be important not only to assess the degree of bottlenecking in the primary host (wheat), but also in alternative hosts. As TriMV populations occur frequently in alternative hosts in addition to their primary host, those alternative hosts become an important part of their evolutionary picture.

Additional research is also needed to fully understand the relationship TriMV has with its vector, the wheat curl mite. For example, the acquisition, inoculation, and latency times are not known. The number of TriMV particles that are transmitted by a single wheat curl mite must be determined to fully appreciate the severity of the bottlenecks that viral populations are undergoing. All of these are important parts of the virus population puzzle that we must resolve in order to completely understand the entirety of the evolutionary forces acting on TriMV and other plant virus populations. A better understanding of plant RNA virus evolution may allow for more effective disease management. Identifying highly conserved, low mutation rate genomic regions should allow targeted control measures, as well as the possibility of more durable resistance to be introduced. This research can help in the never-ending challenge of protecting our agricultural resources against the pressure of continually-evolving plant virus pathogens.

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